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<p>(21) International Application Number: PCT/US99/26825</p> <p>(22) International Filing Date: 12 November 1999 (12.11.99)</p> <p>(30) Priority Data: 60/108,117 12 November 1998 (12.11.98) US</p> <p>(71) Applicant: LIFE TECHNOLOGIES, INC. [US/US]; 9800 Medical Center Drive, Rockville, MD 20850 (US).</p> <p>(72) Inventors: CHU, Yongliang; 10123 Sterling Terrace, Rockville, MD 20850 (US). MASOUD, Malek; 7902 Badenlock Way #301, Gaithersburg, MD 20879 (US). GEBEYEHU, Gulilat; 12400 Rivers Edge Drive, Potomac, MD 20854 (US).</p> <p>(74) Agents: ESMOND, Robert, W. et al.; Sterne, Kessler, Goldstein & Fox P.L.L.C., Suite 600, 1100 New York Avenue, N.W., Washington, DC 20005-3934 (US).</p>		<p>(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</p> <p>Published <i>With international search report.</i></p>
<p>(54) Title: TRANSFECTION REAGENTS</p> <p>(57) Abstract</p> <p>Disclosed are compounds capable of facilitating transport of biologically active agents or substances into cells having general structure (A).</p> <div style="text-align: center;"> $(R_2)_m \left(\begin{array}{c} (R_3)_s \\ \\ Q^* - L - Q^* \\ \quad \\ (A_1)_v \quad (A_2)_w \\ \quad \\ (R_1)_r \quad (R_4)_u \end{array} \right)_q (R_5)_n \quad (A)$ </div>		

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Transfection Reagents

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Background of the Invention

Field of the Invention

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The present invention relates to cationic lipids and compositions of cationic lipids having utility in lipid aggregates for delivery of macromolecules and other compounds into cells.

15 *Related Art*

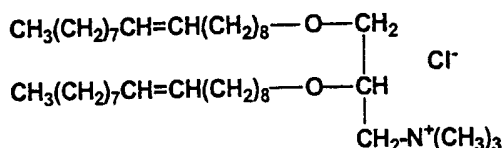
Lipid aggregates such as liposomes have been found to be useful as agents for delivery to introduce macromolecules, such as DNA, RNA, protein, and small chemical compounds such as pharmaceuticals, to cells. In particular, lipid aggregates comprising
20 cationic lipid components have been shown to be especially effective for delivering anionic molecules to cells. In part, the effectiveness of cationic lipids is thought to result from enhanced affinity for cells, many of which bear a net negative charge. Also in part, the net positive charge on lipid aggregates comprising a cationic lipid enables the aggregate to bind polyanions, such as nucleic acids. Lipid aggregates containing DNA
25 are known to be effective agents for efficient transfection of target cells.

The structure of various types of lipid aggregates varies, depending on composition and method of forming the aggregate. Such aggregates include liposomes, unilamellar vesicles, multilamellar vesicles, micelles and the like, having particular sizes in the nanometer to micrometer range. Methods of making lipid aggregates are by now
30 well-known in the art. The main drawback to use of conventional phospholipid-containing liposomes for delivery is that the material to be delivered must be

encapsulated and the liposome composition has a net negative charge which is not attracted to the negatively charged cell surface. By combining cationic lipid compounds with a phospholipid, positively charged vesicles and other types of lipid aggregates can bind DNA, which is negatively charged, can be taken up by target cells, and can transfect target cells. (Felgner, P.L. et al. (1987) Proc. Natl. Acad. Sci. USA 84:7413-7417; Eppstein, D. et al. , U.S. Pat. No. 4,897,355.)

A well-known cationic lipid is N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA). The structure of DOTMA is:

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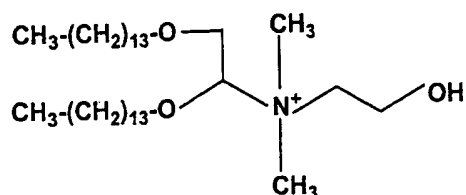


DOTMA by itself or in 1:1 combination with dioleoylphosphatidylethanolamine (DOPE) is formulated into liposomes using standard techniques. Felgner, et al. supra demonstrated that such liposomes provided efficient delivery of nucleic acids to some types of cells. A DOTMA:DOPE (1:1) formulation is sold under the trade name LIPOFECTIN (Life Technologies, Inc., Rockville, MD). Another commercially available cationic lipid is 1,2-bis(oleoyloxy)-3-(trimethylammonia) propane (DOTAP), which differs from DOTMA only in that the oleoyl moieties are linked via ester, rather than ether bonds to the propylamine. A related group of compounds differ from DOTMA and DOTAP in that one of the methyl groups of the trimethylammonium group is replaced by a hydroxyethyl group. Compounds of this type are similar to the Rosenthal Inhibitor (RI) of phospholipase A (Rosenthal, A.F. and Geyer, R.P. (1960) J. Biol. Chem. 235:2202-2206) which has stearoyl esters linked to the propylamine core. The dioleoyl analogs of RI are commonly abbreviated as DORI-ether and DORI-ester, depending on the linkage of the fatty acid moieties to the propylamine core. The hydroxy group can be used as a site for further functionalization.

The dimyristyloxy analog of RI is known as DMRIE. A 1:1 (M/M) DMRIE:cholesterol formulation is sold under the tradename DMRIE-C (Life Technologies, Inc., Rockville, MD). The structure of DMRIE is:

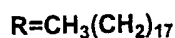
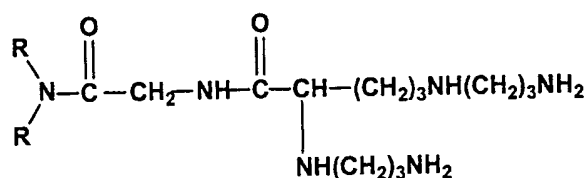
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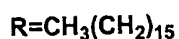
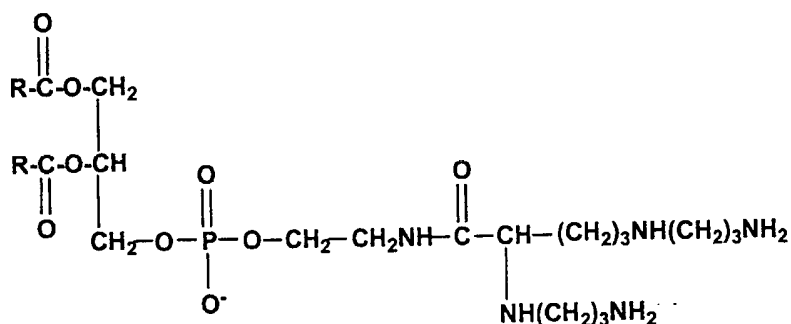
5 Another class of compounds has been disclosed by Behr et al. (1989) Proc. Natl. Acad. Sci. USA 86:6982-6986; EPO publication 0 394 111 (Oct. 24, 1990), in which carboxyspermine has been conjugated to two types of lipids. The structures of 5-carboxyspermylglycine dioctadecylamide (DOGS) is:

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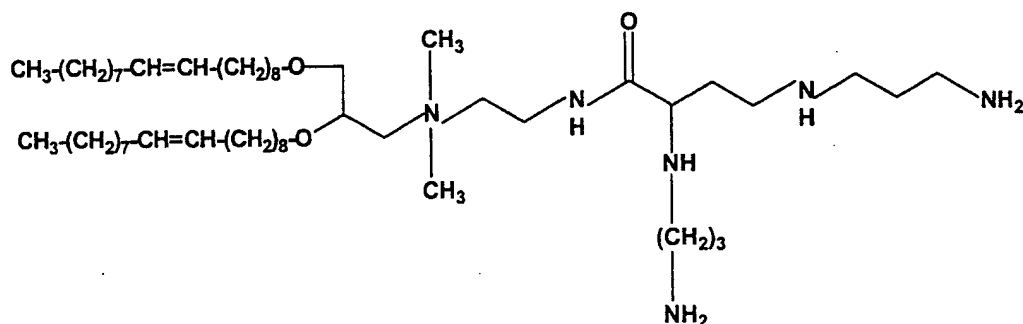
The structure of dipalmitoylphosphatidylethanolamine 5-carboxyspermylamide (DPPES) is:

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Both DOGS and DPPES have been used to coat plasmids, forming a lipid aggregate complex that provides efficient transfection. The compounds are claimed to be more efficient and less toxic than DOTMA for transfection of some cell lines. DOGS is available commercially as TRANSFECTAM™ (Promega, Madison, Wis.).

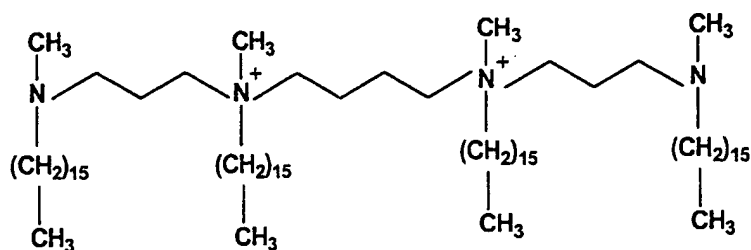
5 Another class of compounds has been also described in which carboxy spermine has been conjugated to lipids via an amide bond (Gebeyehu, G. et al., U.S. Patent No. 5,334,761). These compounds are useful for an efficient delivery of nucleic acids into various cells and also are intermediates for making other such lipids. 2,3-di-oleyloxy-N-[2(spermine-carboxamido)ethyl]-N,N-dimethyl-1-propan-aminium (DOSPA) is available
10 as a 3:1 (w/w) formulation with DOPE under the trade name LipofectAMINE (available from Life Technologies, Inc., Rockville, MD). The structure of DOSPA is as follows:



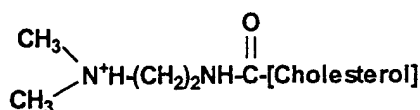
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Lipid compounds with a spermine head group have also been described (Haces, A., et al., U.S. Patent No. 5,674,908). These compounds are especially useful for delivery of nucleic acids into insect cells. A 1:1.5 (M/M) formulation of
20 tetramethyltetrapalmityl spermine (TM-TPS) to DOPE is commercially available under the tradename CellFECTIN (Life Technologies, Inc., Rockville, MD). The structure of TM-TPS is shown below:

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A cationic cholesterol derivative (DC-Chol) has been synthesized and formulated
 5 into liposomes in combination with DOPE. (Gao, X. and Huang, L. (1991) Biochim.
 Biophys. Res. Comm. 179:280-285). The compound's structure is:



10

Liposomes formulated with DC-Chol are said to provide more efficient transfection and
 lower toxicity than DOTMA-containing liposomes for some cell lines.

Lipopolylysine, formed by conjugating polylysine to DOPE, has been reported to
 be especially effective for transfection in the presence of serum, a condition likely to be
 15 encountered *in vivo* (Zhou, X. et al. (1991) Biochim. Biophys. Acta 1065: 8-14).

Despite advances in the field, a need remains for a variety of improved cationic
 lipid compounds. In particular, no single cationic lipid to date has been found to work
 well with all cell types. Since different cell types differ from one another in membrane
 composition, it is not surprising that different compositions and types of lipid aggregates
 20 are effective for different cell types, either for their ability to contact and fuse with target
 cell membranes, or for aspects of the transfer process itself. At present these processes
 are not well understood, consequently the design of effective liposomal precursors is
 largely empirical. Besides content and transfer, other factors are of importance, for
 example, ability to form lipid aggregates suited to the intended purpose, the possibility
 25 of transfecting cells in the presence of serum, toxicity to the target cell, stability as a
 carrier for the compound to be delivered, and ability to function in an *in vivo*
 environment. In addition, lipid aggregates can be improved by broadening the range of

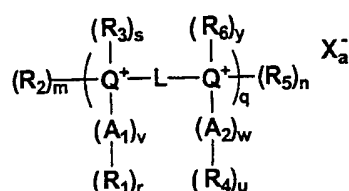
substances which can be delivered to cells. The cationic lipid compounds of the present invention have improved function with respect to several of the foregoing attributes.

Summary of the Invention

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The present invention provides novel cationic lipids according to the general Formula (A):

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wherein

Q is selected from the group consisting of N, O and S;

L is any bivalent organic radical capable of covalently linking each Q, such as C, CH, (CH₂)_i, or {(CH₂)_i - Y - (CH₂)_j}_k, wherein Y is selected from the group consisting of CH₂, an ether, a polyether, an amide, a polyamide, an ester, a sulfide, a urea, a thiourea, a guanidyl, a carbamoyl, a carbonate, a phosphate, a sulfate, a sulfoxide, an imine, a carbonyl, and a secondary amino group and wherein Y is optionally substituted by -X₁-L'-X₂-Z or -Z;

20

R₁ - R₆, independently of one another, are selected from the group consisting of H, -(CH₂)_p-D-Z, an alkyl, an alkenyl, an aryl, and an alkyl or alkyl ether optionally substituted by one or more of an alcohol, an aminoalcohol, an amine, an amide, an ether, a polyether, a polyamide, an ester, a mercaptan, an alkylthio, a urea, a thiourea, a guanidyl, or a carbamoyl group, and wherein at least one of R₁, R₃, R₄ and R₆ is a straight chain or branched, cyclic, alkyl, alkenyl, alkynyl or aryl group; and any one or more of R₁, R₃, R₄ and R₆ may optionally be covalently linked with each other, with Y or with L when L is C or CH to form a cyclic moiety;

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Z is selected from the group consisting of amine, spermyl, carboxyspermyl, guanidyl, spermidinyl, putricinyl, diaminoalkyl, pyridyl, piperidinyl, pyrrolidinyl, polyamine, amino acid, peptide, and protein;

X_1 and X_2 , independently of one another, are selected from the group consisting of NH, O, S, alkylene, and arylene;

L' is selected from the group consisting of alkylene, alkenylene, alkynylene, arylene, alkylene ether, and polyether;

D is Q or a bond;

A_1 and A_2 , independently of one another, are selected from the group consisting of CH_2O , CH_2S , CH_2NH , $C(O)$, $C(NH)$, $C(S)$ and $(CH_2)_i$;

X is a physiologically acceptable anion;

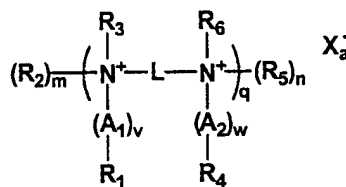
m, n, r, s, u, v, w and y are 0 or 1, with the proviso that when both m and n are 0 at least one of r, s, u and y is other than 0;

i, j, k, l, p and t are from 0 to about 100;

q is an integer from 1 to about 1000; and

a is the number of positive charge divided by the valence of the anion.

Further, the present invention provides novel cationic lipids according to the general Formula (B):



wherein

L is $(CH_2)_l$ or $\{(CH_2)_i - Y - (CH_2)_j\}_k$ wherein Y is selected from the group consisting of an ether, a polyether, an amide, a polyamide, an ester, a sulfide, a urea, a thiourea, a guanidyl, a carbamoyl, a carbonate, and a secondary amino group;

$R_1 - R_6$, independently of one another, are selected from the group consisting of H, $-(CH_2)_p-Z$, an alkyl, an alkenyl, an aryl, and an alkyl or an alkyl ether optionally substituted by one or more of an alcohol, an aminoalcohol, an amine, an amide, an ether,

a polyether, a polyamide, an ester, a mercaptan, a urea, a thiourea, a guanidyl, or a carbamoyl group, and at least one of R_1 , R_3 , R_4 and R_6 is a straight chain or branched, cyclic, alkyl, alkenyl, alkynyl or aryl group, preferably having from about 2 to 100, preferably 4 to 75, more preferably 6 to 64, more preferably 8 to 50, more preferably 8 to 40, more preferably 8 to 30, more preferably 6 to 30, more preferably 4 to 30, more preferably 2 to 30, and most preferably 8 to about 24 carbon atoms, and any one or more of R_1 , R_3 , R_4 and/or R_6 may optionally be covalently linked with each other to form a cyclic moiety;

Z is selected from the group consisting of amine, spermyl, carboxyspermyl, guanidyl, spermidinyl, putricinyl, diaminoalkyl, pyridyl, piperidinyl, pyrrolidinyl, polyamine, amino acid, amino acid derivative, peptide, and protein;

A_1 and A_2 , independently of one another, are selected from the group consisting of CH_2O , CH_2S , CH_2NH , $\text{C}(\text{O})$, $\text{C}(\text{NH})$, $\text{C}(\text{S})$ and $(\text{CH}_2)_t$;

X is a physiologically acceptable anion, such as the halide anions, chloride, bromide, and iodide as well as acetate, sulfate, trifluoroacetate, etc.;

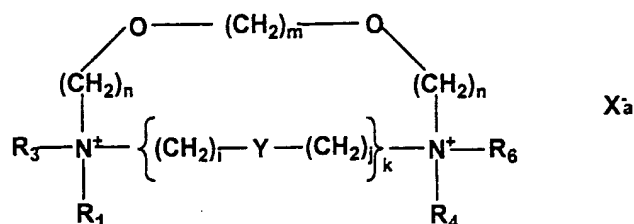
m, n, v and w are 0 or 1;

i, j, k, l, p and t are integers from 1 to about 100, more preferably 1 to 50, more preferably 1 to 25, more preferably 1 to 15, more preferably 1 to 10 and most preferably 1 to about 4;

q is an integer from 1 to about 1000, preferably from 1 to about 500, more preferably from 1 to about 250, more preferably from 1 to about 100, more preferably from 1 to about 50, more preferably from 1 to about 25, more preferably from 1 to about 12, most preferably from 1 to about 6; and

a is the number of positive charges divided by the valence of the anion, wherein when m and n are 0, then a is 0.

Also, the present invention provides novel cationic lipids according to the Formula (C):



wherein

- 5 Y is selected from the group consisting of CH₂, an ether, a polyether, an amide, a polyamide, an ester, a sulfide, a urea, a thiourea, a guanidyl, a carbamoyl, a carbonate, a phosphate, a sulfate, a sulfoxide, an imine, a carbonyl, and a secondary amino group and wherein Y is optionally substituted by -X₁-L'-X₂-Z or -Z;

- R₁, R₃, R₄ and R₆, independently of one another, are selected from the group
 10 consisting of H, -(CH₂)_p-D-Z, an alkyl, an alkenyl, an aryl, and an alkyl or an alkyl ether optionally substituted by one or more of an alcohol, an aminoalcohol, an amine, an amide, an ether, a polyether, a polyamide, an ester, a mercaptan, an alkylthio, a urea, a thiourea, a guanidyl, or a carbamoyl group, and at least one of R₁, R₃, R₄ and R₆ is a
 15 straight chain or branched, cyclic, alkyl, alkenyl, alkynyl or aryl group, most preferably having from about 8 to about 24 carbon atoms, and R₁, R₃, R₄ and R₆ may optionally be
 covalently linked with each other or with Y, to form a cyclic moiety;

 Z is selected from the group consisting of amine, spermiyl, caboxyspermiyl, guanidyl, spermidinyl, putricinyl, diaminoalkyl, pyridyl, piperidinyl, pyrrolidinyl, polyamine, amino acid, peptide, and protein;

- 20 X₁ and X₂, independently of one another, are selected from the group consisting of NH, O, S, alkylene, and arylene;

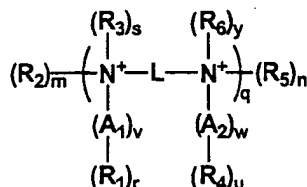
 L' is selected from the group consisting of alkylene, alkenylene, alkynylene, arylene, alkylene ether, and polyether;

 D is Q or a bond;

- 25 m and n are 0 or 1; and

 i, j, k, l and p are integers from 1 to about 10.

Further, the present invention provides compounds or polycations according to the Formula (D):



wherein

5 L is C, CH, (CH₂)_i, or {(CH₂)_i-Y-(CH₂)_j}_k, wherein Y is selected from the group consisting of CH₂, an ether, a polyether, an amide, a polyamide, an ester, a sulfide, a urea, a thiourea, a guanidyl, a carbamoyl, a carbonate, a phosphate, a sulfate, a sulfoxide, an imine, a carbonyl, and a secondary amino group and wherein Y is optionally substituted by -X₁-L'-X₂-Z or -Z;

10 R₁ - R₆, independently of one another, are selected from the group consisting of H, -(CH₂)_p-D-Z, an alkyl, an alkenyl, an aryl, and an alkyl or an alkyl ether optionally substituted by one or more of an alcohol, an aminoalcohol, an amine, an amide, an ether, a polyether, a polyamide, an ester, a mercaptan, an alkylthio, a urea, a thiourea, a guanidyl, or a carbamoyl group, and wherein at least one of R₁, R₃, R₄ and R₆ is a straight
15 chain or branched, cyclic, alkyl, alkenyl, alkynyl or aryl groups, preferably having from about 2 to about 30 carbon atoms, more preferably from 8 to 24 carbon atoms;

Z is selected from the group consisting of amine, spermyl, carboxyspermyl, guanidyl, spermidinyl, putricinyl, diaminoalkyl, pyridyl, piperidinyl, pyrrolidinyl, polyamine, amino acid, amino acid derivative, peptide, and protein;

20 X₁ and X₂, independently of one another, are selected from the group consisting of NH, O, S, alkylene and arylene;

L' is selected from the group consisting of alkylene, alkenylene, alkynylene, arylene, alkylene ether, and polyether;

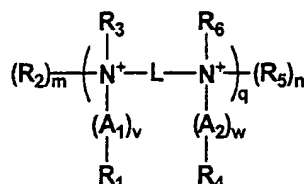
A₁ and A₂, independently of one another, are selected from the group consisting
25 of CH₂O, CH₂S, CH₂NH, C(O), C(NH), C(S) and (CH₂)_i;

m, n, r, s, u, v, w and y are 0 or 1, with the proviso that when both m and n are 0 at least one of r, s, u and y is other than 0;

i, j, k, l, p and t are integers from 0 to about 100; and

q is an integer from 1 to about 1000.

Also, the present invention provides compounds or polycations according to the Formula (E):



wherein

L is $(CH_2)_l$ or $\{(CH_2)_i - Y - (CH_2)_j\}_k$, wherein Y is selected from the group consisting of CH_2 , an ether, a polyether, an amide, a polyamide, an ester, a sulfide, a urea, a thiourea, a guanidyl, a carbamoyl, a carbonate, and a secondary amino group;

10 $R_1 - R_6$, independently of one another, are selected from the group consisting of H, $-(CH_2)_p-Z$, an alkyl, an alkenyl, an aryl, and an alkyl or an alkyl ether optionally substituted by one or more of an alcohol, an aminoalcohol, an amine, an amide, an ether, a polyether, a polyamide, an ester, a mercaptan, a urea, a thiourea, a guanidyl, or a carbamoyl group, and at least one of R_1 , R_3 , R_4 and R_6 is a straight chain or branched,
15 cyclic, alkyl, alkenyl, alkenyl or aryl group, preferably having from about 2 to about 30 carbon atoms, more preferably having from about 8 to about 24 carbon atoms;

Z is selected from the group consisting of amine, spermiyl, carboxyspermiyl, guanidyl, spermidinyl, putricinyl, diaminoalkyl, pyridyl, piperidinyl, pyrrolidinyl, polyamine, amino acid, amino acid derivative, peptide, and protein;

20 A_1 and A_2 , independently of one another, are selected from the group consisting of CH_2O , CH_2S , CH_2NH , $C(O)$, $C(NH)$, $C(S)$ and $(CH_2)_t$;

m, n, v and w are 0 or 1;

i, j, k, l, p and t are integers from 1 to about 100; and

q is an integer from 1 to about 1000.

25 Also, the present invention provides novel compounds falling within the scope of the above formulae.

The compounds of the invention are useful, either alone or in combination with other lipid aggregate-forming components (e.g., DOPE, DOPC or cholesterol) for formulation into liposomes or other lipid aggregates. Such aggregates are polycationic,

able to form stable complexes with anionic macromolecules, such as nucleic acids. The lipid aggregate macromolecular complex interacts with cells making the polyanionic macromolecule available for absorption and uptake by the cell.

5 The present invention provides a lipid aggregate comprising one or more of the compounds of the present invention. Preferably, the lipid aggregate comprises at least one lipid aggregate-forming compound. Preferably, the lipid aggregate-forming compound is selected from the group consisting of DOPE, DOPC and cholesterol.

10 The compounds of the present invention may also be conjugated to or mixed with or used in conjunction with a variety of useful molecules and substances such as proteins, peptides, growth factors and the like to enhance cell-targeting, uptake, internalization, nuclear targeting and expression.

This invention also includes lipid aggregates comprising one or more compounds of the present invention or mixtures thereof. Such lipid aggregates may be combined with one or more aggregate-forming components and/or transfection enhancers.

15 The transfection methods of the present invention employing the compounds or compositions (such as those described above) of the present invention or mixtures thereof can be applied to *in vitro* and *in vivo* transfection of cells, particularly to transfection of eukaryotic cells or tissues including animal cells, human cells, insect cells, plant cells, avian cells, fish cells, mammalian cells and the like.

20 Accordingly, the present invention provides a method for introducing a polyanion into a cell or cells, wherein the method comprises forming a liposome from a positively charged compound according to the invention, contacting the liposome with polyanion to form a positively-charged polyanion-liposome complex and incubating the complex with a cell or cells.

25 The methods of this invention can be used to generate transfected cells or tissues which express useful gene products. The methods of this invention can also be used as a step in the production of transgenic animals. The methods of this invention are useful in any therapeutic method requiring introducing of nucleic acids into cells or tissues. In particular, these methods are useful in cancer treatment, in *in vivo* and *ex vivo* gene therapy, and in diagnostic methods. See, for example, U.S. Patent No. 5,589,466 to
30 Felgner, et al. and U.S. Patent Application No. 08/450,555 filed on May 25, 1995 to

Jessee, et al. The transfection compounds or compositions of this invention can be employed as research reagents in any transfection of cells or tissues done for research purposes. Nucleic acids that can be transfected by the methods of this invention include DNA and RNA from any source comprising natural bases or non-natural bases, and include those encoding and capable of expressing therapeutic or otherwise useful proteins in cells or tissues, those which inhibit expression of nucleic acids in cells or tissues, those which inhibit enzymatic activity or activate enzymes, those which catalyze reactions (ribozymes), and those which function in diagnostic assays.

The compounds, compositions and methods provided herein can also be readily adapted in view of the disclosure herein to introduce biologically active macromolecules or substances other than nucleic acids, including, among others, polyamines, polyamine acids, polypeptides, proteins, biotin, and polysaccharides into cells. Other useful materials for example, therapeutic agents, diagnostic materials and research reagents, can be introduced into cells by the methods of this invention. In a preferred aspect, any nucleic acid vector may be delivered to or into a cell by the present invention.

Accordingly, the present invention provides a method for introducing a biologically active substance into a cell, wherein the method comprises forming a liposome of a compound according to the invention and a biologically active substance and incubating the liposome with a cell or cell culture.

The invention also relates to compositions comprising the compounds of the invention and one or more additional components selected from the group consisting of nucleic acids, cells, buffers, culture media, biologically active substance, neutral lipids, and transfection enhancers, preferably a nucleic acid.

This invention also includes transfection kits which include one or more of the compounds or compositions of the present invention or mixtures thereof. Particularly, the invention provides a kit comprising one or more of the compounds of the present invention and at least one additional component selected from the group consisting of a cell, cells, a cell culture media, a nucleic acid, a transfection enhancer and instructions for transfecting a cell or cells.

The invention also relates to intermediates and methods for using such intermediates for making the compounds or compositions of the invention. The

invention also relates to the compositions, compounds or components obtained by the interaction of materials (intermediates, compounds, lipids etc.) used in the synthesis methods of the invention.

Other preferred embodiments of the present invention will be apparent to one of ordinary skill in the art in view of the following drawings and description of the invention.

Brief Description of the Drawings

Figure 1 is a graph showing the transfection of HEK-293 cells with cationic transfection reagents.

Figure 2 is a graph showing transfection of COS-7 cells with cationic transfection reagents.

Figure 3 is a graph showing transfection of CHO-K1 cells with cationic transfection reagents.

Figure 4 is a graph showing transfection of HeLa cells with cationic transfection reagents.

Detailed Description of the Preferred Embodiments

20

The present invention relates to cationic lipids and compositions of cationic lipids having utility in lipid aggregates for delivery of macromolecules and other compounds into cells. The compounds can be used alone or in combination with other compounds to prepare liposomes and other lipid aggregates suitable for transfection or delivery of compounds to target cells, either *in vitro* or *in vivo*.

25

The compounds of the present invention are preferably polycationic and preferably thus form highly stable complexes with various anionic macromolecules, particularly polyanions such as nucleic acids. These compounds have the property, when dispersed in water, of forming lipid aggregates which associate strongly, via their cationic portion, with polyanions. By using an excess of cationic charges relative to the anionic

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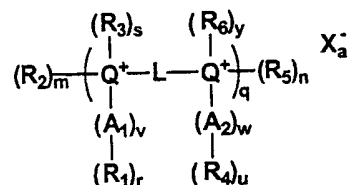
compound, the polyanion-lipid complexes may be adsorbed on cell membranes, thereby facilitating uptake of the desired compound by the cells.

The present invention also relates to intermediates for preparing the compound and compositions of the invention.

- 5 More specifically, the present invention relates to a cationic lipid for transfection which has a greater transfection efficiency than commercially available products in the three most common cell types used in expression research (CHO-K1, COS-7, and HEK293) making it useful for high throughput applications; and which has a simple to use protocol as defined by the fact that no additional reagents are required (e.g., such as
- 10 LipofectAMINE PLUS Reagent available from Life Technologies, Inc., Rockville, MD), no removal of serum and therefore no media changes are required, and the DNA/lipid complex do not need to be removed from the cells prior to assay.

The compounds according to the present invention have the Formula (A):

15



wherein

Q is selected from the group consisting of N, O and S;

- L is any bivalent organic radical capable of covalently linking each Q, such as C, CH, (CH₂)_i, or {(CH₂)_i-Y-(CH₂)_j}_k, wherein Y is selected from the group consisting of CH₂, an ether, a polyether, an amide, a polyamide, an ester, a sulfide, a urea, a thiourea, a guanidyl, a carbamoyl, a carbonate, a phosphate, a sulfate, a sulfoxide, an imine, a carbonyl, and a secondary amino group and wherein Y is optionally substituted by -X₁-L'-X₂-Z or -Z;
- 20

- 25 R₁ - R₆, independently of one another, are selected from the group consisting of H, -(CH₂)_p-D-Z, an alkyl, an alkenyl, an aryl, and an alkyl or alkyl ether optionally substituted by one or more of an alcohol, an aminoalcohol, an amine, an amide, an ether, a polyether, a polyamide, an ester, a mercaptan, an alkylthio, a urea, a thiourea, a guanidyl, or a carbamoyl group, and wherein at least one of R₁, R₃, R₄ and R₆ is a straight

chain or branched, cyclic, alkyl, alkenyl, alkynyl or aryl group; and R_1 and R_4 or R_3 and R_6 may optionally be covalently linked with each other, with Y or with L when L is C or CH to form a cyclic moiety;

Z is selected from the group consisting of amine, spermyl, carboxyspermyl, guanidyl, spermidinyl, putricinyl, diaminoalkyl, pyridyl, piperidinyl, pyrrolidinyl, polyamine, amino acid, peptide, and protein;

X_1 and X_2 , independently of one another, are selected from the group consisting of NH, O, S, alkylene, and arylene;

L' is selected from the group consisting of alkylene, alkenylene, alkynylene, arylene, alkylene ether, and polyether;

D is Q or a bond;

A_1 and A_2 , independently of one another, are selected from the group consisting of CH_2O , CH_2S , CH_2NH , $\text{C}(\text{O})$, $\text{C}(\text{NH})$, $\text{C}(\text{S})$ and $(\text{CH}_2)_i$;

X is a physiologically acceptable anion;

m, n, r, s, u, v, w and y are 0 or 1, with the proviso that when both m and n are 0 at least one of r, s, u and y is other than 0;

i, j, k, l, p and t are integers from 0 to about 100;

q is an integer from 1 to about 1000; and

a is the number of positive charge divided by the valence of the anion.

Preferably the alkyl ether optionally substituted by one or more alcohol groups comprises a carbohydrate. Preferably, the carbohydrate is selected from the group consisting of galactose, fructose, glucose, maltose, sucrose, cellobiose, lactose, mannose, glucopyranose, mannopyranose and galactopyranose.

Preferably, i, j, k, l, p and t are integers independently selected from 1 to 100, more preferably from 1 to 50, more preferably 1 to 25, more preferably 1 to 15, more preferably 1 to 10 and most preferably 1 to about 4. Preferably, l, b and c are integers from 1 to about 4, i and j are integers from about 2 to about 3 and k is an integer from 1 to about 3.

Preferably, q is an integer from 1 to about 500, more preferably from 1 to about 250, more preferably from 1 to about 100, more preferably from 1 to about 50, more

preferably from 1 to about 25, more preferably from 1 to about 12, most preferably from 1 to about 6.

Preferably, at least one of R_1 , R_3 , R_4 and R_6 is a straight chain or branched, cyclic, alkyl, alkenyl, alkynyl or aryl group having from about 2 to 100, preferably 4 to 75, more
5 preferably 6 to 64, more preferably 8 to 50, more preferably 8 to 40, more preferably 8 to 30, more preferably 6 to 30, more preferably 4 to 30, and most preferably 8 to about 24 carbon atoms.

In all aspects of the invention, most suitable R_1 and R_4 groups, which can be the same or different, preferably the same, are C_{6-30} hydrocarbon radicals derived from fatty
10 acids or activated derivatives thereof, such as fatty acyl chlorides. Thus, typical R_1 and R_4 groups are C_{6-30} alkyl or alkenyl groups.

Preferably, R_1 , R_2 , R_3 , R_4 , R_5 and R_6 , independently of one another, are selected from the group consisting of H, C_1 - C_8 alkyl, alkenyl, aryl, and alkyl optionally substituted by one or more of an alcohol, an amide, an ether, a polyether, a polyamide, an ester, a
15 mercaptan, a urea, a thiourea, a guanidyl, or a carbamoyl group, and at least one of R_1 , R_3 , R_4 and R_6 is a straight chain or branched, cyclic, alkyl, alkenyl, alkynyl or aryl group having from about 8 to about 24 carbon atoms.

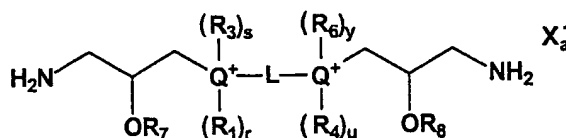
Preferably Q is N.

Preferably, Y is selected from the group consisting of CH_2 , O, S and NH.

Useful compounds falling within the scope of the above formula (A) include
20 compounds having the following formulae:

(A1)

25



wherein

Q and L are as defined above;

R_1 , R_3 , R_4 and R_6 , independently of one another, are selected from the group
30 consisting of H and a C_1 - C_8 alkyl, alkenyl, aryl, and alkyl optionally substituted by one

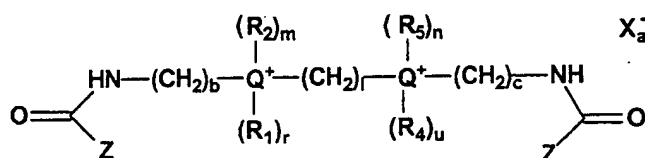
or more of an alcohol, an amine, an amide, an ether, a polyether, a polyamide, an ester, a mercaptan, a urea, a thiourea, a guanidyl, or a carbamoyl group;

r, s, u and y are 0 or 1; and

R₇ and R₈ are independently H or a carbohydrate;

5

(A2)



10 wherein

Q is as defined above;

R₁, R₂, R₄ and R₅, independently of one another, are selected from the group consisting of H and a C₁-C₈ alkyl, alkenyl, aryl, and alkyl optionally substituted by one or more of an alcohol, an amine, an amide, an ether, a polyether, a polyamide, an ester, a mercaptan, a urea, a thiourea, a guanidyl, or a carbamoyl group;

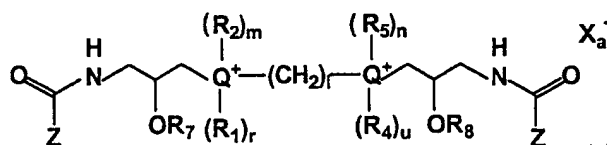
Z is selected from the group consisting of spermyl, spermidyl, amino acid, peptidyl, diaminoalkyl, and polyamine;

m, n, r and u are 0 or 1; and

l, b and c are integers independently selected from 1 to about 4;

20

(A3)



25 wherein

Q, R₁, R₄, m, n, r and u are as defined above;

R₂ and R₅, independently of one another, are selected from the group consisting of H and a C₁-C₈ alkyl, alkenyl, aryl, and alkyl optionally substituted by one or more of

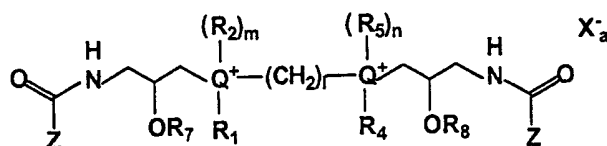
an alcohol, an amine, an amide, an ether, a polyether, a polyamide, an ester, a mercaptan, a urea, a thiourea, a guanidyl, or a carbamoyl group;

Z is selected from the group consisting of spermyl, spermidyl, amino acid, peptidyl, diaminoalkyl, and polyamine;

- 5 R_7 and R_8 are independently H or a carbohydrate; and
1 is an integer from 1 to about 4;

(A4)

10



wherein

Q is as defined above, preferably N;

- at least one of R_1 and R_4 are straight chain or branched, cyclic, alkyl, alkenyl,
15 alkynyl or aryl groups having from about 8 to about 24 carbon atoms;

R_2 and R_5 , independently of one another, are selected from the group consisting of H and a C_1 - C_8 alkyl, alkenyl, aryl, and alkyl optionally substituted by one or more of an alcohol, an amine, an amide, an ether, a polyether, a polyamide, an ester, a mercaptan, a urea, a thiourea, a guanidyl, or a carbamoyl group;

- 20 Z is selected from the group consisting of spermyl, spermidyl, amino acid, peptidyl, diaminoalkyl, and polyamine;

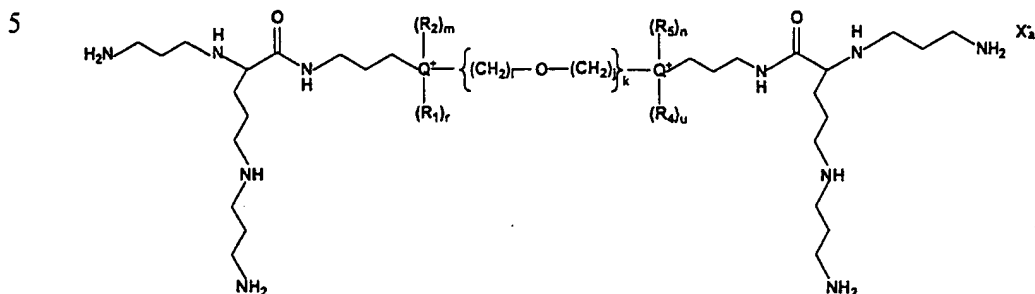
R_7 and R_8 are independently H or a carbohydrate, preferably H; m and n are as defined above; and

1 is an integer from 1 to about 4;

25

30

(A5)



wherein

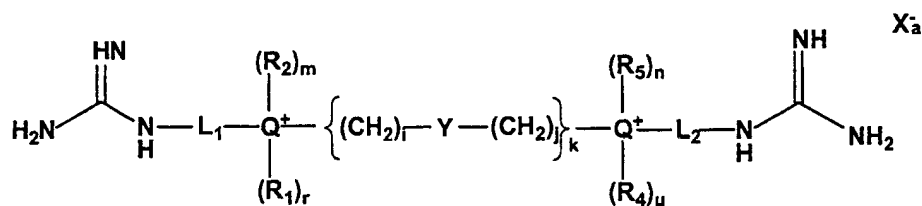
Q, R₁, R₄, r, u, m and n are as defined above;

R₂ and R₅, independently of one another, are selected from the group consisting of H and a C₁-C₈ alkyl, alkenyl, aryl, and alkyl optionally substituted by one or more of an alcohol, an amine, an amide, an ether, a polyether, a polyamide, an ester, a mercaptan, a urea, a thiourea, a guanidyl, or a carbamoyl group;

i and j are integers from about 2 to about 3; and

k is an integer from 1 to about 3;

15 (A6)



wherein

20 Q, R₁, R₄, r, u, m and n are as defined above;

R₂ and R₅, independently of one another, are selected from the group consisting of H and a C₁-C₈ alkyl, alkenyl, aryl, and alkyl optionally substituted by one or more of an alcohol, an amine, an amide, an ether, a polyether, a polyamide, an ester, a mercaptan, a urea, a thiourea, a guanidyl, or a carbamoyl group;

25 i and j are integers from about 2 to about 3;

-21-

k is an integer from 1 to about 3;

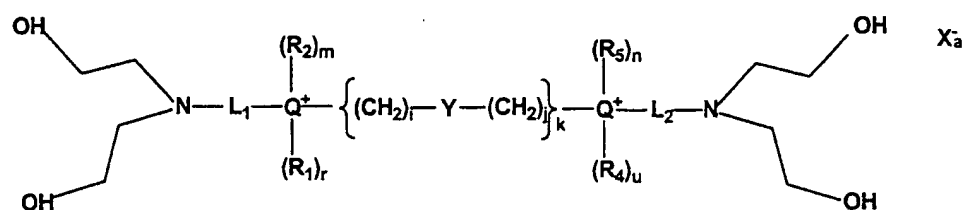
L_1 and L_2 , independently from one another, are an alkylene or an alkylene ether;

and

Y is selected from the group consisting of CH_2 , O, S and NH;

5

(A7)



10 wherein

Q, R_1 , R_4 , r, u, m and n are as defined above;

R_2 and R_3 , independently of one another, are selected from the group consisting of H and a C_1 - C_8 alkyl, alkenyl, aryl, and alkyl optionally substituted by one or more of an alcohol, an amine, an amide, an ether, a polyether, a polyamide, an ester, a mercaptan, a urea, a thiourea, a guanidyl, or a carbamoyl group;

15

i and j are integers from about 2 to about 3;

k is an integer from 1 to about 3;

L_1 and L_2 , independently from one another, are an alkylene or an alkylene ether;

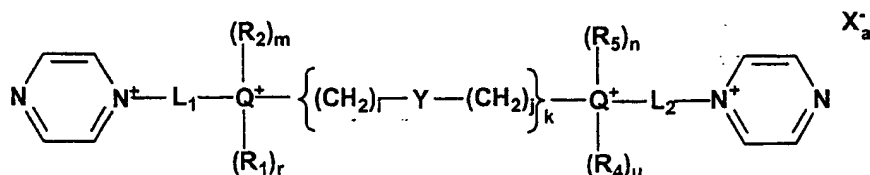
and

20

Y is selected from the group consisting of CH_2 , O, S and NH;

(A8)

25



wherein

Q, R₁, R₄, r, u, m and n are as defined above;

R₂ and R₅, independently of one another, are selected from the group consisting of H and a C₁ - C₈ alkyl, alkenyl, aryl, and alkyl optionally substituted by one or more of
 5 an alcohol, an amine, an amide, an ether, a polyether, a polyamide, an ester, a mercaptan, a urea, a thiourea, a guanidyl, or a carbamoyl group;

i and j are integers from about 2 to about 3;

k is an integer from 1 to about 3;

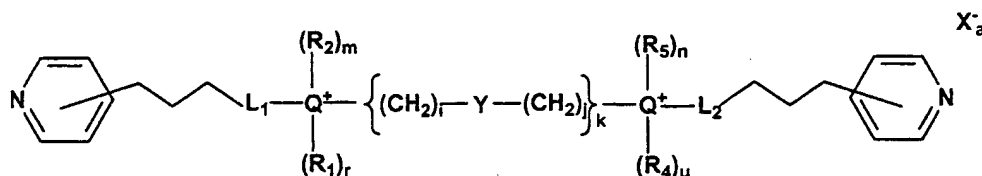
L₁ and L₂, independently from one another, are an alkylene or an alkylene ether;

10 and

Y is selected from the group consisting of CH₂, O, S and NH;

(A9)

15



wherein

Q, R₁, R₂, r, u, m and n are as defined above;

20 R₂ and R₅, independently of one another, are selected from the group consisting of H and a C₁ - C₈ alkyl, alkenyl, aryl, and alkyl optionally substituted by one or more of an alcohol, an amine, an amide, an ether, a polyether, a polyamide, an ester, a mercaptan, a urea, a thiourea, a guanidyl, or a carbamoyl group;

i and j are integers from about 2 to about 3;

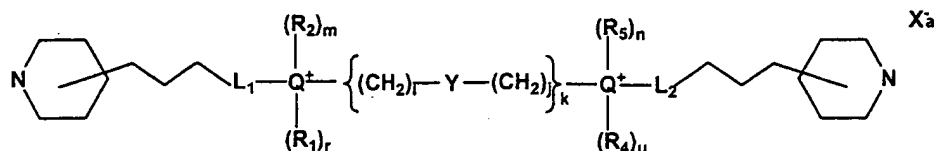
25 k is an integer from 1 to about 3;

L₁ and L₂, independently from one another, are an alkylene or an alkylene ether;

and

Y is selected from the group consisting of CH₂, O, S and NH; and

(A10)



wherein

5 Q, R₁, R₄, r, u, m, and n are as defined above;

R₂ and R₅, independently of one another, are selected from the group consisting of H and a C₁ - C₈ alkyl, alkenyl, aryl, and alkyl optionally substituted by one or more of an alcohol, an amine, an amide, an ether, a polyether, a polyamide, an ester, a mercaptan, a urea, a thiourea, a guanidyl, or a carbamoyl group;

10 i and j are integers from about 2 to about 3;

k is an integer from 1 to about 3;

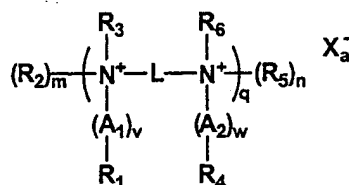
L₁ and L₂, independently from one another, are an alkylene or an alkylene ether;

and

Y is selected from the group consisting of CH₂, O, S and NH.

15

Also, compounds of the present invention have the Formula (B):



20 wherein

L is (CH₂)_i or {(CH₂)_i - Y - (CH₂)_j}_k, wherein Y is selected from the group consisting of CH₂, an ether, a polyether, an amide, a polyamide, an ester, a sulfide, a urea, a thiourea, a guanidyl, a carbamoyl, a carbonate, and a secondary amino group;

25 R₁ - R₆, independently of one another, are selected from the group consisting of H, -(CH₂)_p-Z, an alkyl, an alkenyl, an aryl, and an alkyl or alkyl ether optionally substituted by one or more of an alcohol, an aminoalcohol, an amine, an amide, an ether, a polyether, a polyamide, an ester, a mercaptan, a urea, a thiourea, a guanidyl, or a

carbamoyl group, and at least one of R_1 , R_3 , R_4 and R_6 is a straight chain or branched, cyclic, alkyl, alkenyl, alkynyl or aryl group, and any one or more of R_1 , R_4 , R_3 and R_6 may optionally be covalently linked with each other to form a cyclic moiety;

5 Z is selected from the group consisting of amine, spermyl, carboxyspermyl, guanidyl, spermidinyl, putricinyl, diaminoalkyl, pyridyl, piperidinyl, pyrrolidinyl, polyamine, amino acid, peptide, and protein;

A_1 and A_2 , independently of one another, are selected from the group consisting of CH_2O , CH_2S , CH_2NH , $\text{C}(\text{O})$, $\text{C}(\text{NH})$, $\text{C}(\text{S})$ and $(\text{CH}_2)_t$;

X is a physiologically acceptable anion;

10 m, n, v and w are 0 or 1;

i, j, k, l, p and t are integers from 1 to about 100;

q is an integer from 1 to about 1000; and

a is the number of positive charge divided by the valence of the anion, wherein when m and n are 0, then a is 0.

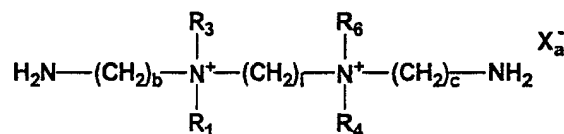
15 Preferably, R_1 - R_6 , i, j, k, l, p, t, q, b and c are as defined with reference to Formula (A).

Preferably, Y is selected from the group consisting of CH_2 , O, S and NH.

Useful compounds falling within the scope of the Formula (B) include compounds having the following formulae:

20

(B1)



25 wherein

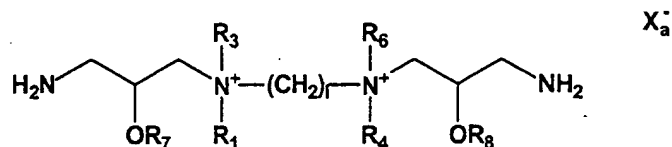
R_1 , R_3 , R_4 and R_6 , independently of one another, are selected from the group consisting of H and a C_1 - C_8 alkyl, alkenyl, aryl, and alkyl optionally substituted by one or more of an alcohol, an amine, an amide, an ether, a polyether, a polyamide, an ester, a mercaptan, a urea, a thiourea, a guanidyl, or a carbamoyl group, and at least one of R_1 ,

-25-

R_3 , R_4 and R_6 is a straight chain or branched, cyclic, alkyl, alkenyl, alkynyl or aryl group having from about 8 to about 24 carbon atoms; and

l , b and c are integers independently selected from 1 to about 4;

5 (B2)



wherein

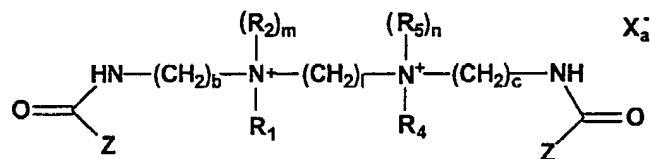
- 10 R_1 , R_3 , R_4 and R_6 , independently of one another, are selected from the group consisting of H and a C_1 - C_8 alkyl, alkenyl, aryl, and alkyl optionally substituted by one or more of an alcohol, an amine, an amide, an ether, a polyether, a polyamide, an ester, a mercaptan, a urea, a thiourea, a guanidyl, or a carbamoyl group, and at least one of R_1 , R_3 , R_4 and R_6 is a straight chain or branched, cyclic, alkyl, alkenyl, alkynyl or aryl group
- 15 having from about 8 to about 24 carbon atoms;

R_7 and R_8 are independently H or a carbohydrate; and

l is an integer from 1 to about 4;

(B3)

20



wherein

- 25 R_1 , R_2 , R_4 and R_5 , independently of one another, are selected from the group consisting of H and a C_1 - C_8 alkyl, alkenyl, aryl, and alkyl optionally substituted by one or more of an alcohol, an amine, an amide, an ether, a polyether, a polyamide, an ester, a mercaptan, a urea, a thiourea, a guanidyl, or a carbamoyl group, and at least one of R_1 ,

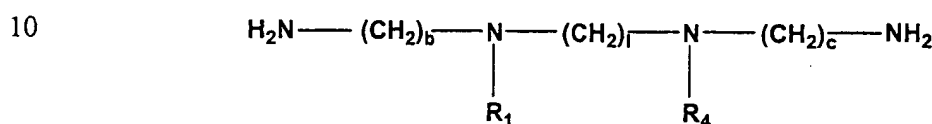
R₂, R₄ and R₅ is a straight chain or branched, cyclic, alkyl, alkenyl, alkynyl or aryl group having from about 8 to about 24 carbon atoms;

Z is selected from the group consisting of spermyl, spermidyl, amino acid, peptidyl, diaminoalkyl, and polyamine;

5 m and n are 0 or 1;and

1, b and c are integers independently selected from 1 to about 4;

(B4)

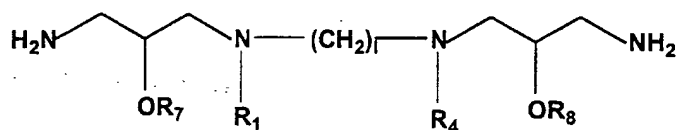


wherein

at least one of R₁ and R₄ is a straight chain or branched, cyclic, alkyl, alkenyl, alkynyl or aryl group having from about 8 to about 24 carbon atoms; and

1, b and c are integers independently selected from 1 to about 4;

(B5)

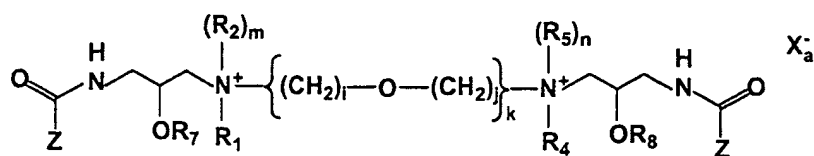


wherein

20 at least one of R₁ and R₄ are straight chain or branched, cyclic, alkyl, alkenyl, alkynyl or aryl groups having from about 8 to about 24 carbon atoms; R₇ and R₈ are independently hydrogen or a carbohydrate, preferably hydrogen; and

l is an integer from 1 to about 4;

25 (B6)



-27-

wherein

Z is as defined above;

at least one of R_1 and R_4 is a straight chain or branched, cyclic, alkyl, alkenyl, alkynyl or aryl group having from about 8 to about 24 carbon atoms;

5 R_2 and R_5 , independently of one another, are selected from the group consisting of H and a C_1 - C_8 alkyl, alkenyl, aryl, and alkyl optionally substituted by one or more of an alcohol, an amine, an amide, an ether, a polyether, a polyamide, an ester, a mercaptan, a urea, a thiourea, a guanidyl, or a carbamoyl group;

R_7 and R_8 are independently H or a carbohydrate;

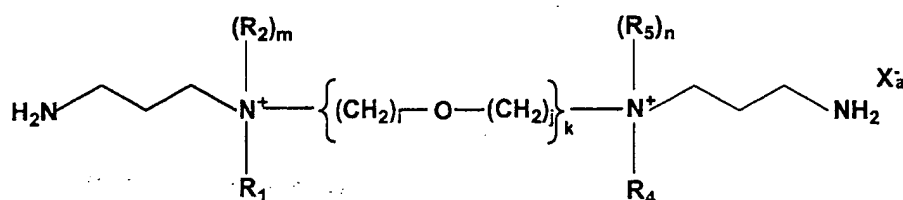
10 m and n are 0 or 1;

i and j are integers from about 2 to about 3; and

k is an integer from 1 to about 3;

(B7)

15



wherein

at least one of R_1 and R_4 is a straight chain or branched, cyclic, alkyl, alkenyl, alkynyl or aryl group having from about 8 to about 24 carbon atoms;

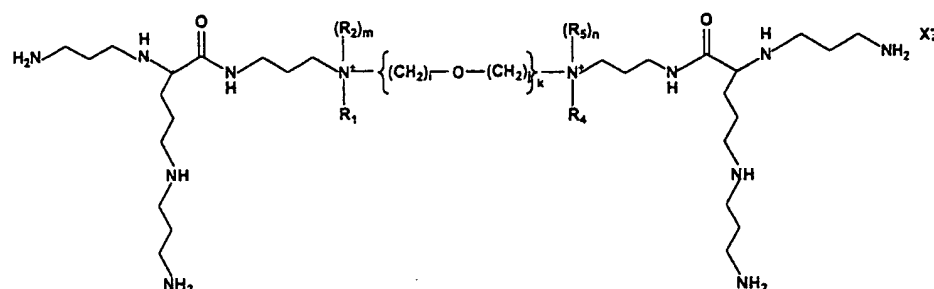
20 R_2 and R_5 , independently of one another, are selected from the group consisting of H and a C_1 - C_8 alkyl, alkenyl, aryl, and alkyl optionally substituted by one or more of an alcohol, an amine, an amide, an ether, a polyether, a polyamide, an ester, a mercaptan, a urea, a thiourea, a guanidyl, or a carbamoyl group;

25 m and n are 0 or 1;

i and j are integers from about 2 to about 3; and

k is an integer from 1 to about 3;

(B8)



5 wherein

at least one of R_1 and R_4 is a straight chain or branched, cyclic, alkyl, alkenyl, alkynyl or aryl group having from about 8 to about 24 carbon atoms;

R_2 and R_5 , independently of one another, are selected from the group consisting of H and a C_1 - C_8 alkyl, alkenyl, aryl, and alkyl optionally substituted by one or more of
 10 an alcohol, an amine, an amide, an ether, a polyether, a polyamide, an ester, a mercaptan, a urea, a thiourea, a guanidyl, or a carbamoyl group;

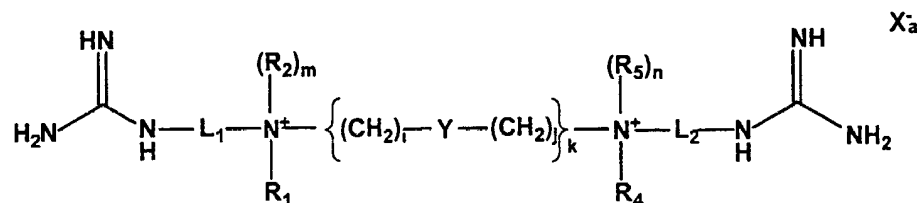
m and n are 0 or 1;

i and j are integers from about 2 to about 3; and

k is an integer from 1 to about 3;

15

(B9)



20

wherein

at least one of R_1 and R_4 is a straight chain or branched, cyclic, alkyl, alkenyl, alkynyl or aryl group having from about 8 to about 24 carbon atoms;

R_2 and R_5 , independently of one another, are selected from the group consisting
 25 of H and a C_1 - C_8 alkyl, alkenyl, aryl, and alkyl optionally substituted by one or more of

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an alcohol, an amine, an amide, an ether, a polyether, a polyamide, an ester, a mercaptan, a urea, a thiourea, a guanidyl, or a carbamoyl group;

m and n are 0 or 1;

i and j are integers from about 2 to about 3;

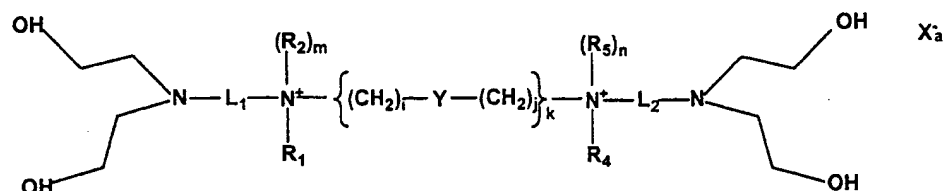
5 k is an integer from 1 to about 3;

L_1 and L_2 , independently from one another, are an alkylene or an alkylene ether;

and

Y is selected from the group consisting of CH_2 , O, S and NH;

10 (B10)



wherein

15 at least one of R_1 and R_4 is a straight chain or branched, cyclic, alkyl, alkenyl, alkynyl or aryl group having from about 8 to about 24 carbon atoms;

R_2 and R_5 , independently of one another, are selected from the group consisting of H and a $C_1 - C_8$ alkyl, alkenyl, aryl, and alkyl optionally substituted by one or more of an alcohol, an amine, an amide, an ether, a polyether, a polyamide, an ester, a mercaptan,

20 a urea, a thiourea, a guanidyl, or a carbamoyl group;

m and n are 0 or 1;

i and j are integers from about 2 to about 3;

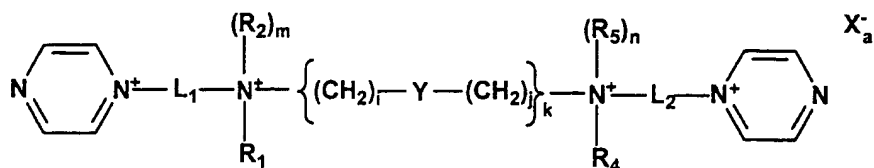
k is an integer from 1 to about 3;

L_1 and L_2 , independently from one another, are an alkylene or an alkylene ether;

25 and

Y is selected from the group consisting of CH_2 , O, S and NH;

(B11)



wherein

- 5 at least one of R_1 and R_4 is a straight chain or branched, cyclic, alkyl, alkenyl, alkynyl or aryl group having from about 8 to about 24 carbon atoms;

R_2 and R_5 , independently of one another, are selected from the group consisting of H and a $C_1 - C_8$ alkyl, alkenyl, aryl, and alkyl optionally substituted by one or more of an alcohol, an amine, an amide, an ether, a polyether, a polyamide, an ester, a mercaptan,

- 10 a urea, a thiourea, a guanidyl, or a carbamoyl group;

m and n are 0 or 1;

i and j are integers from about 2 to about 3;

k is an integer from 1 to about 3;

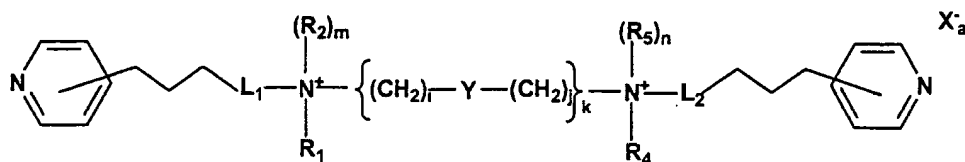
L_1 and L_2 , independently from one another, are an alkylene or an alkylene ether;

- 15 and

Y is selected from the group consisting of CH_2 , O, S and NH;

(B12)

20



wherein

at least one of R_1 and R_4 is a straight chain or branched, cyclic, alkyl, alkenyl, alkynyl or aryl group having from about 8 to about 24 carbon atoms;

- 25 R_2 and R_5 , independently of one another, are selected from the group consisting of H and a $C_1 - C_8$ alkyl, alkenyl, aryl, and alkyl optionally substituted by one or more of an alcohol, an amine, an amide, an ether, a polyether, a polyamide, an ester, a mercaptan, a urea, a thiourea, a guanidyl, or a carbamoyl group;

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m and n are 0 or 1;

i and j are integers from about 2 to about 3;

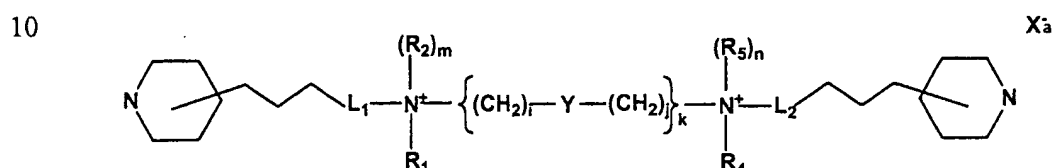
k is an integer from 1 to about 3;

L₁ and L₂, independently from one another, are an alkylene or an alkylene ether;

5 and

Y is selected from the group consisting of CH₂, O, S and NH; and

(B13)



wherein

at least one of R₁ and R₄ is a straight chain or branched, cyclic, alkyl, alkenyl, alkynyl or aryl group having from about 8 to about 24 carbon atoms;

15 R₂ and R₅, independently of one another, are selected from the group consisting of H and a C₁ - C₈ alkyl, alkenyl, aryl, and alkyl optionally substituted by one or more of an alcohol, an amine, an amide, an ether, a polyether, a polyamide, an ester, a mercaptan, a urea, a thiourea, a guanidyl, or a carbamoyl group;

m and n are 0 or 1;

i and j are integers from about 2 to about 3;

20 k is an integer from 1 to about 3;

L₁ and L₂, independently from one another, are an alkylene or an alkylene ether;

and

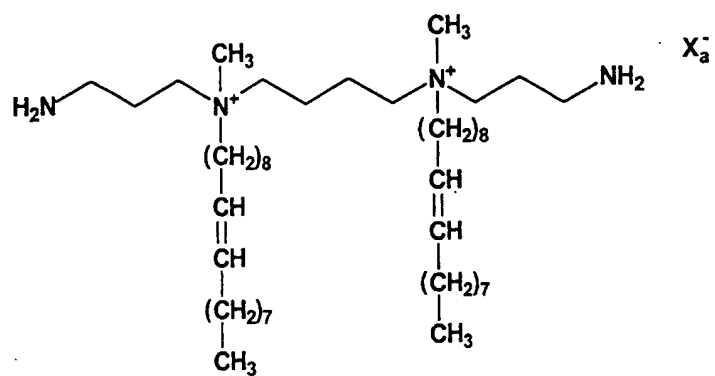
Y is selected from the group consisting of CH₂, O, S and NH.

25 In each of formulae (B1) through (B13) preferably R₁ and R₄ are each C₆₋₃₀ alkyl or alkenyl, more preferably C₈₋₂₄ alkyl or alkenyl, and R₂ and R₅ or R₃ and R₆ are each hydrogen or C₁₋₈ alkyl.

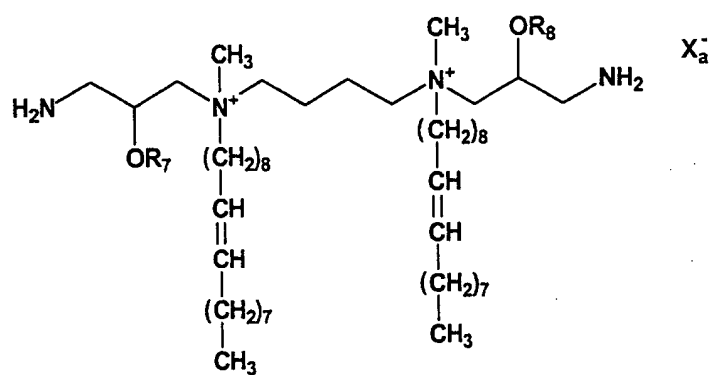
Specific compounds within the scope of the invention include the following examples. R₇ and R₈ in the formulae are independently H or a carbohydrate, preferably

30 H.

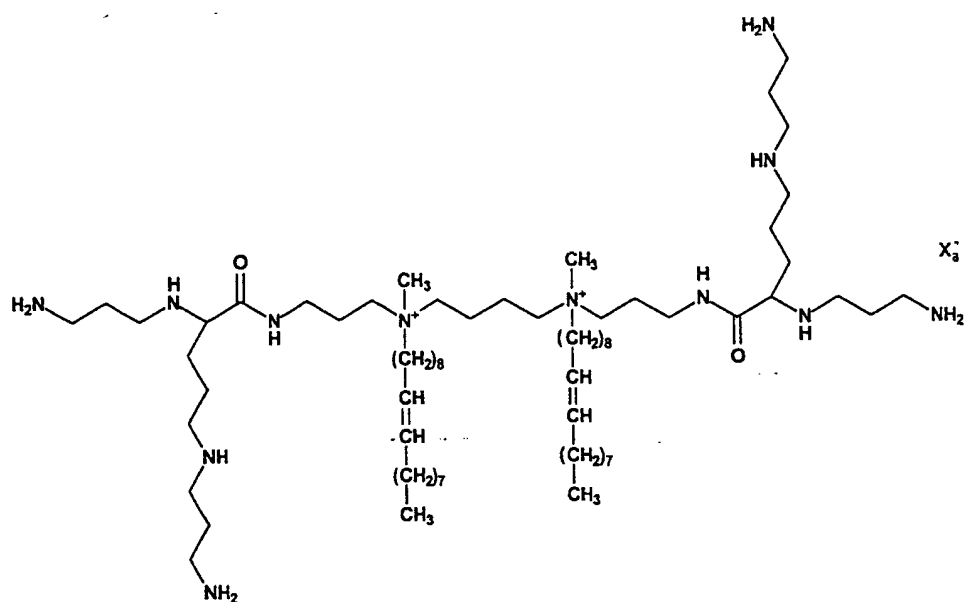
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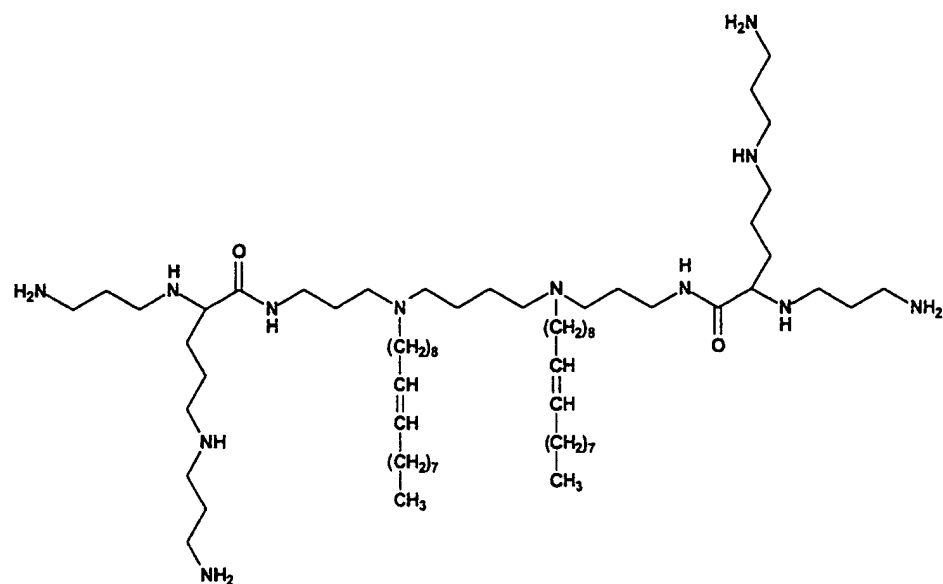


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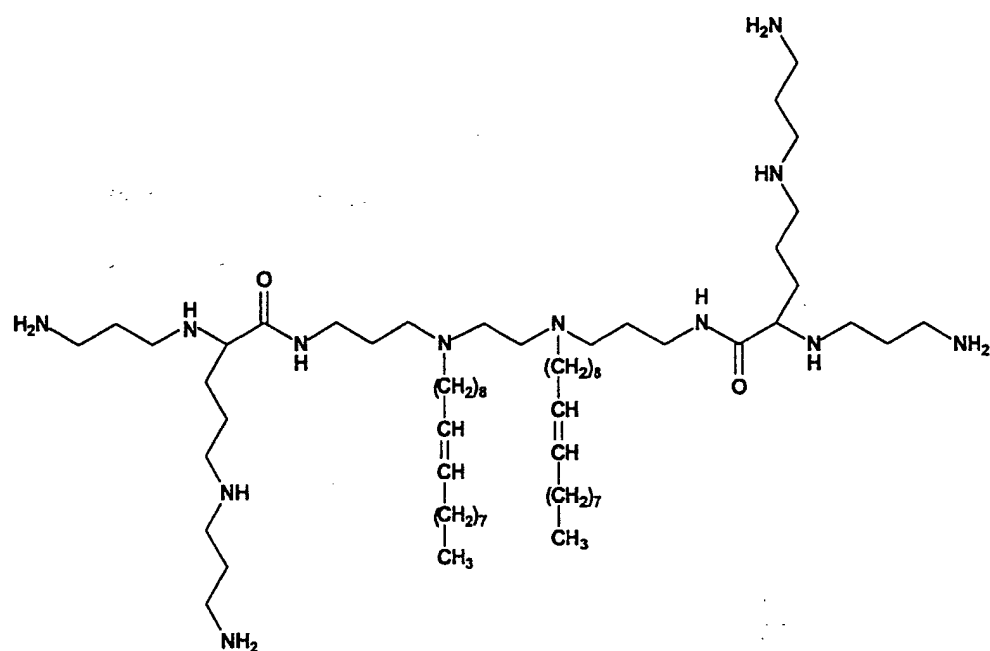


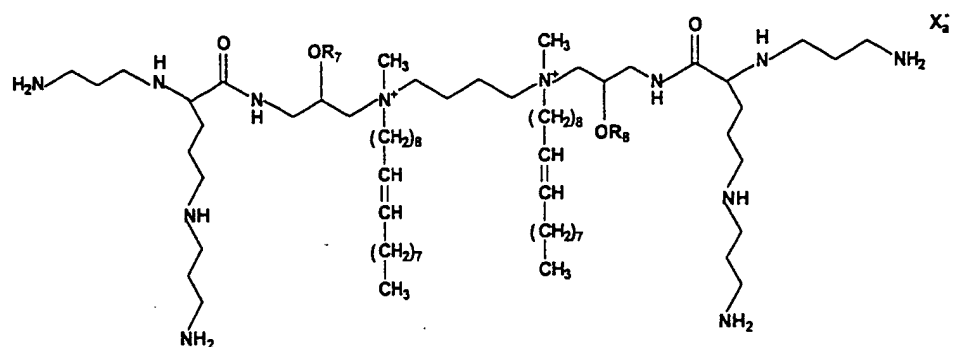
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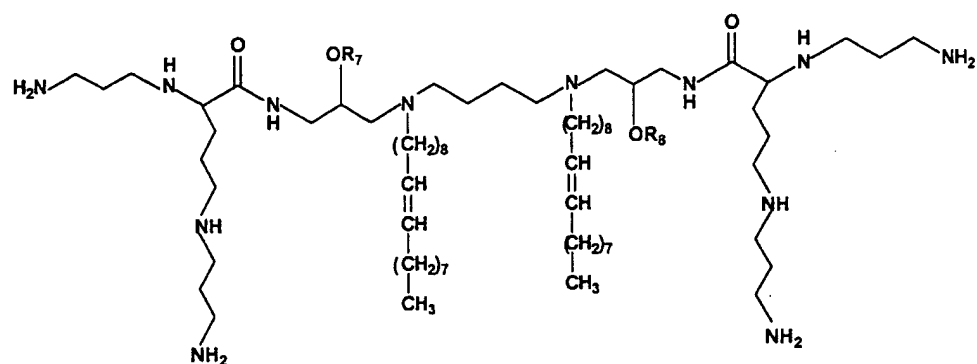


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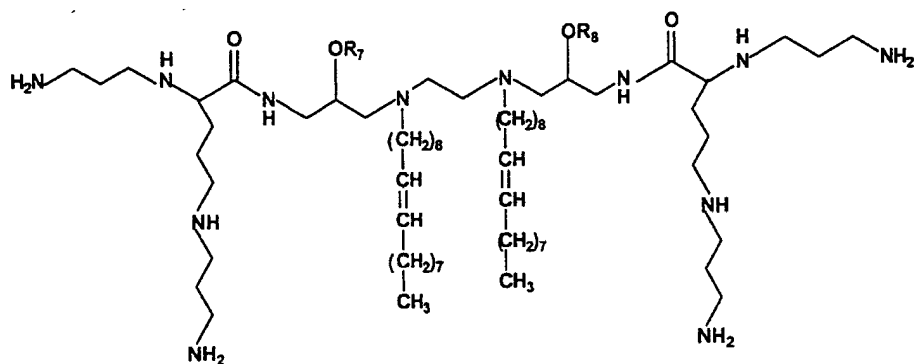




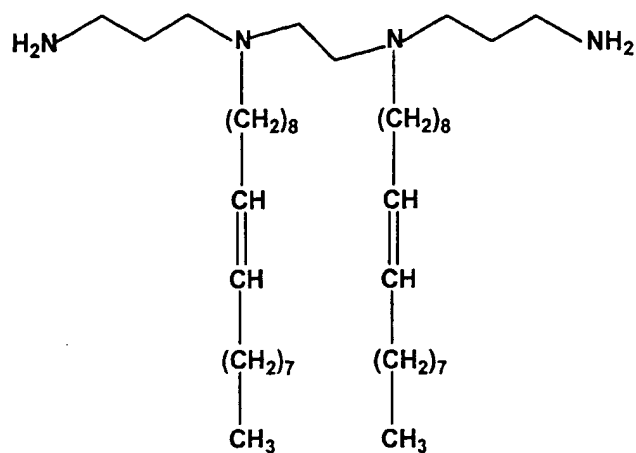
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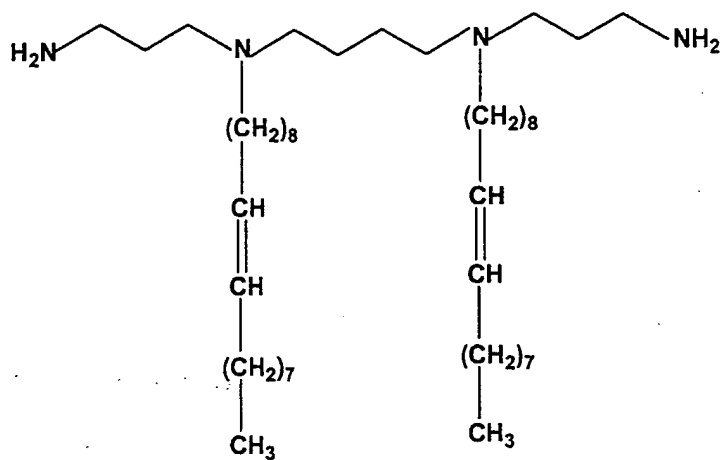
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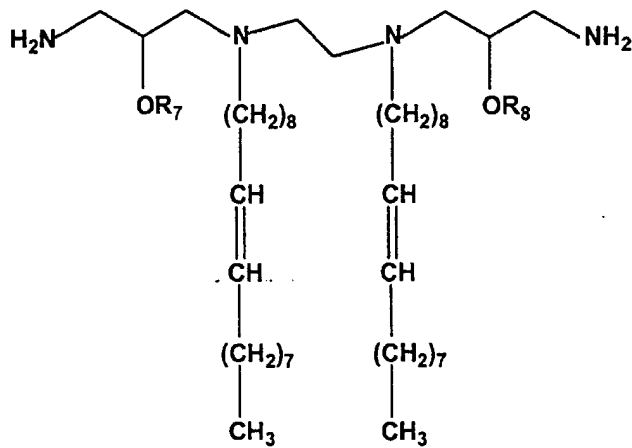
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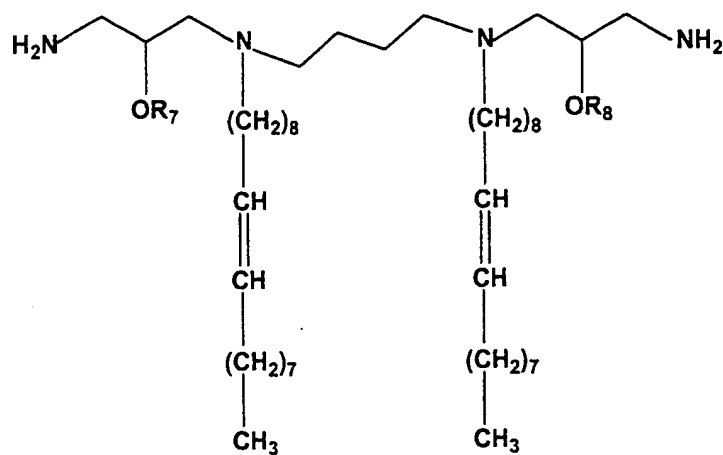
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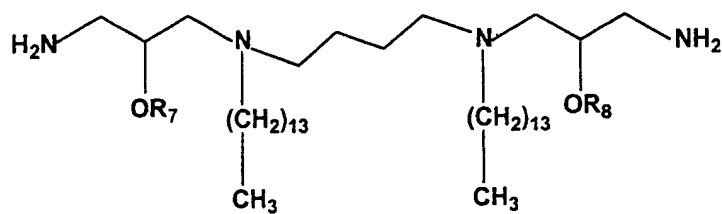
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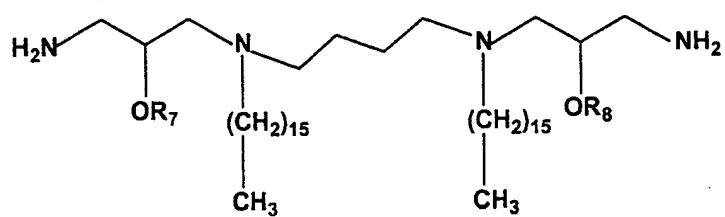
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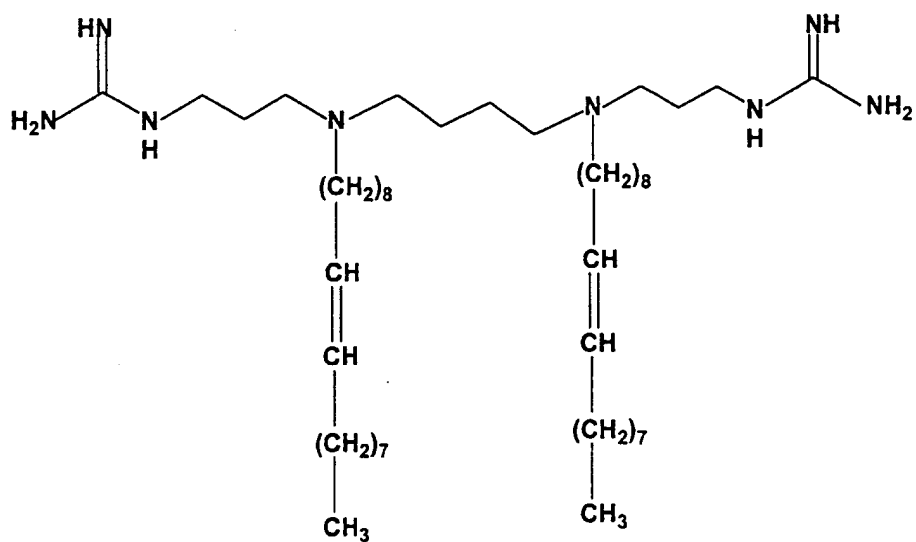
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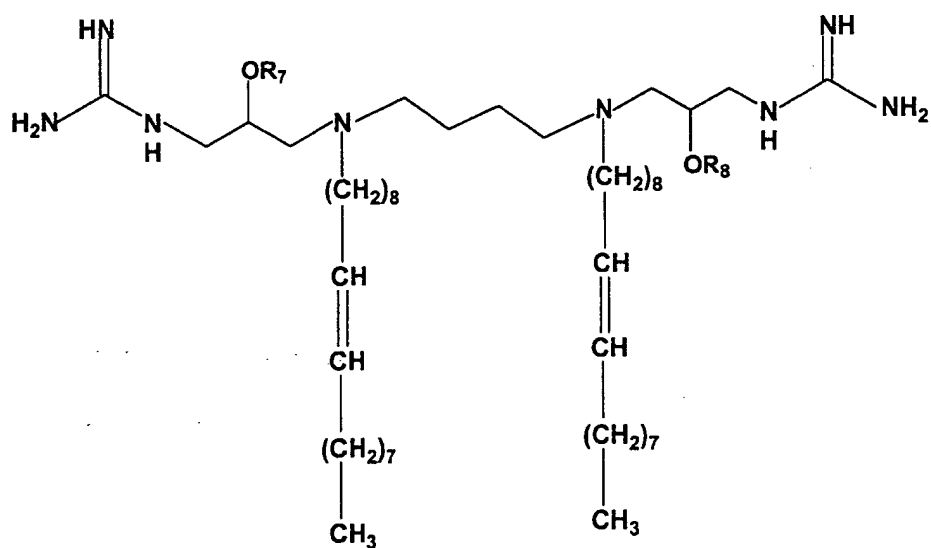
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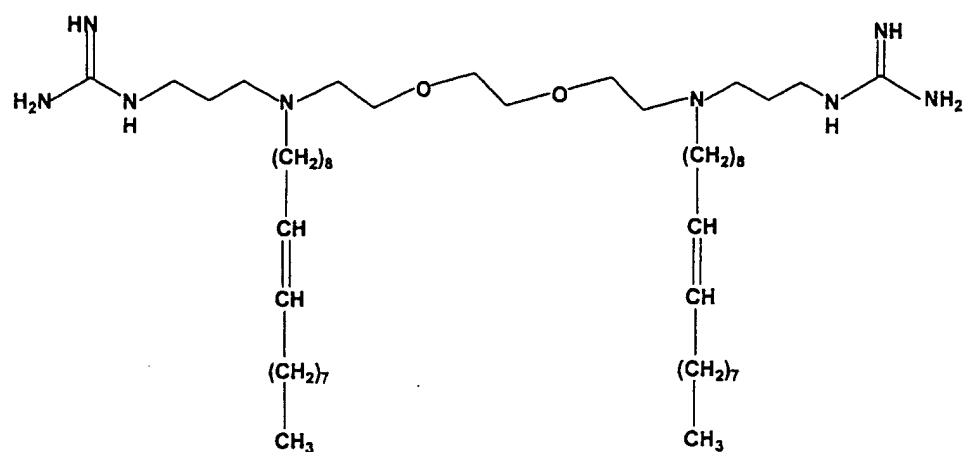
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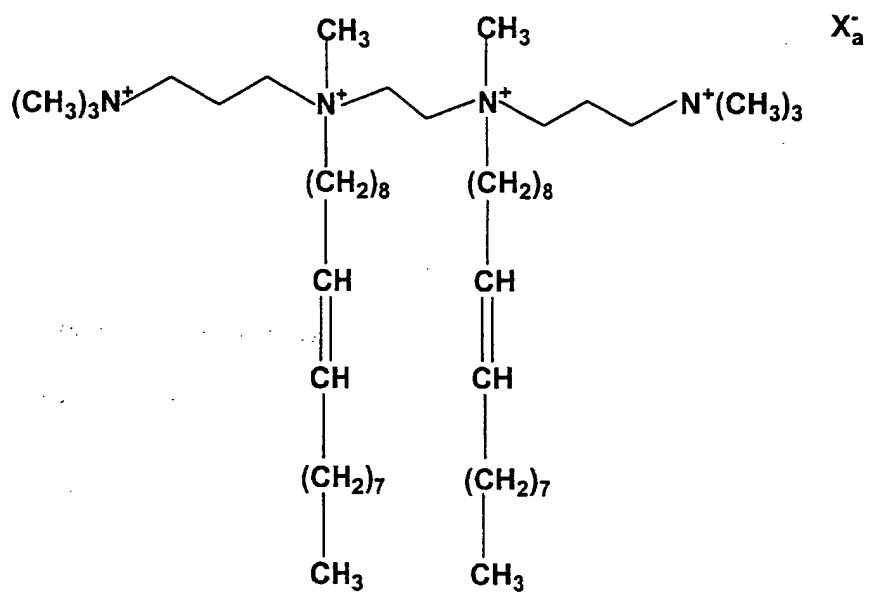
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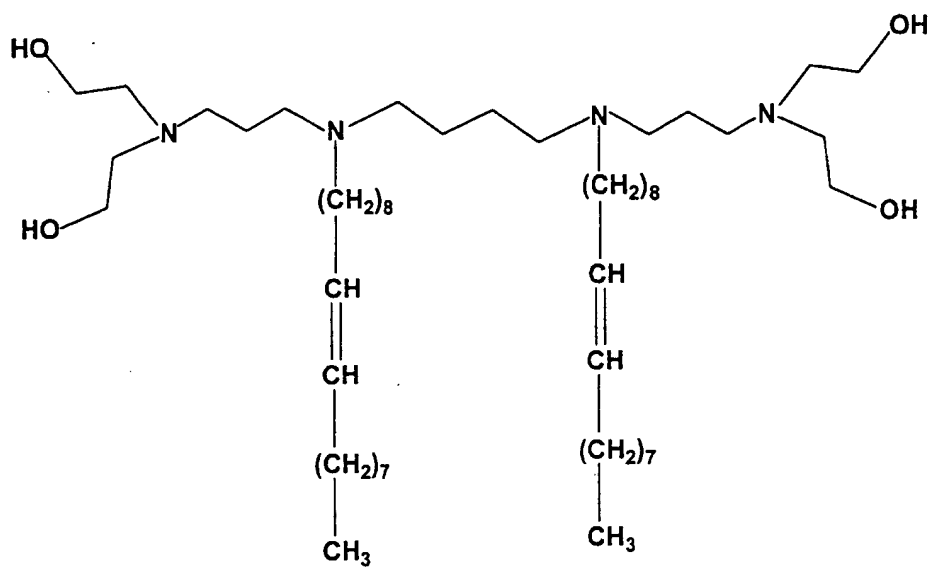


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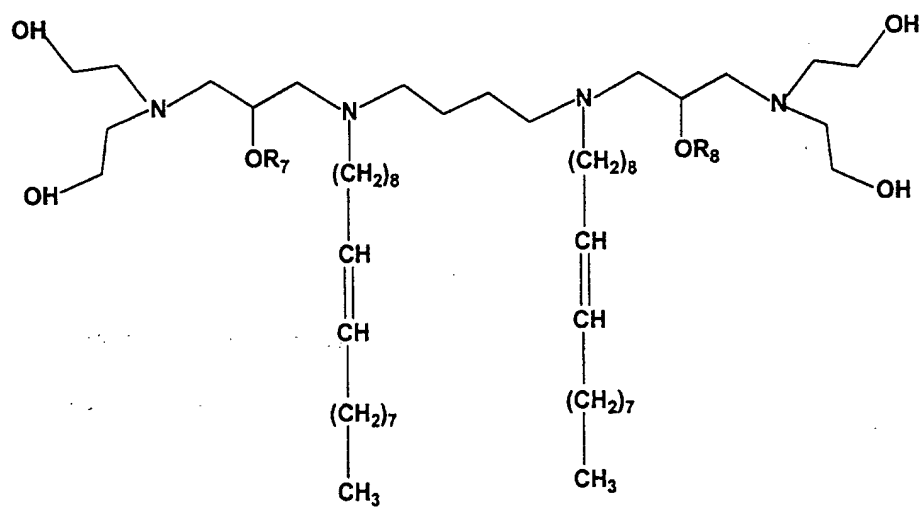


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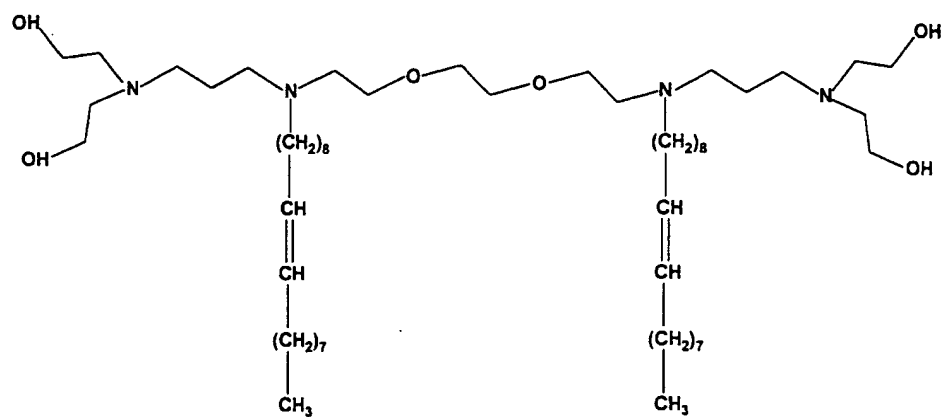
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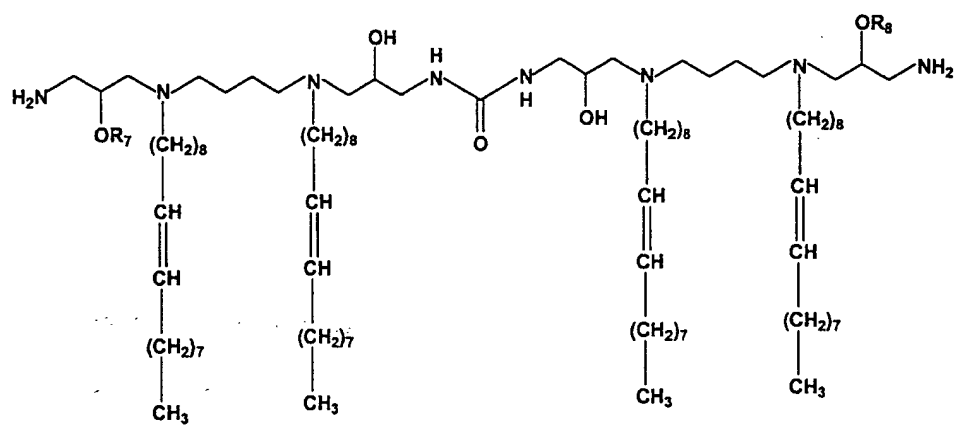
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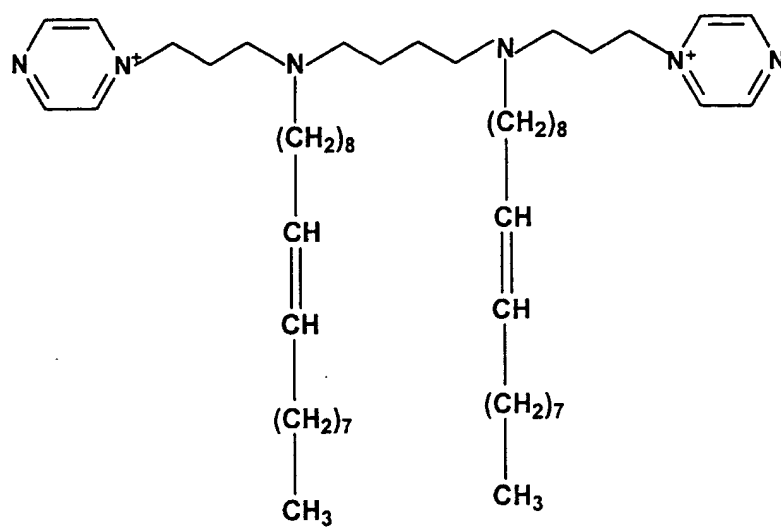


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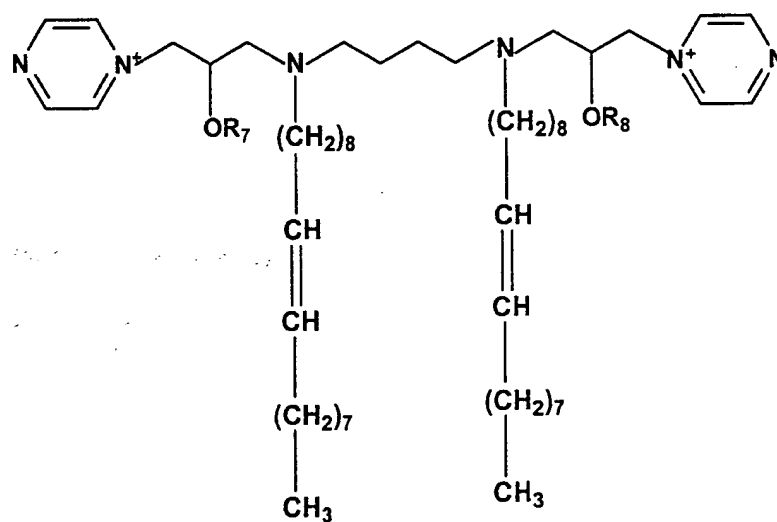


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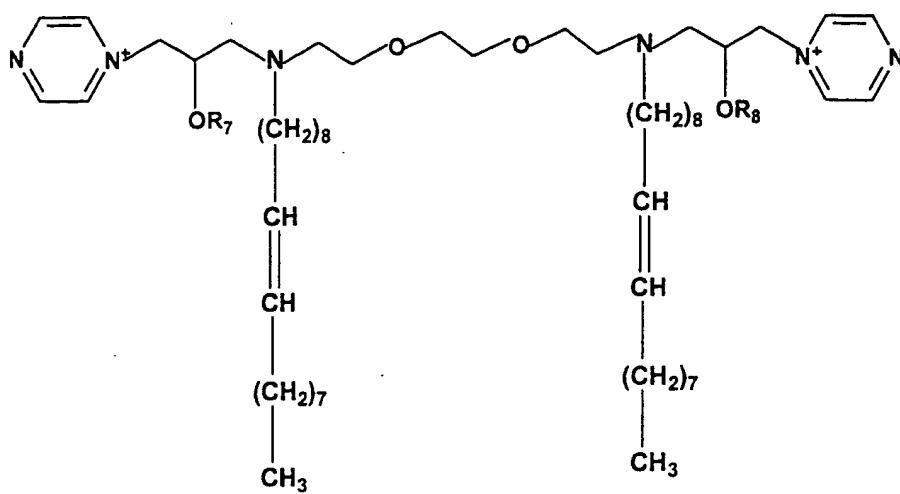


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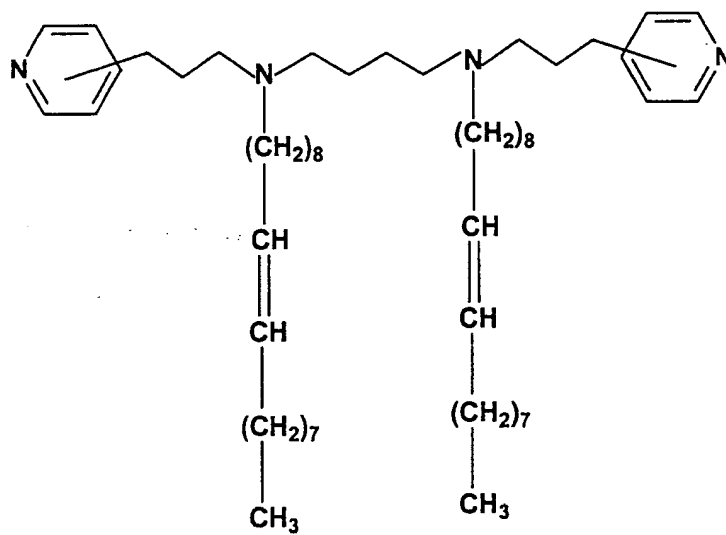


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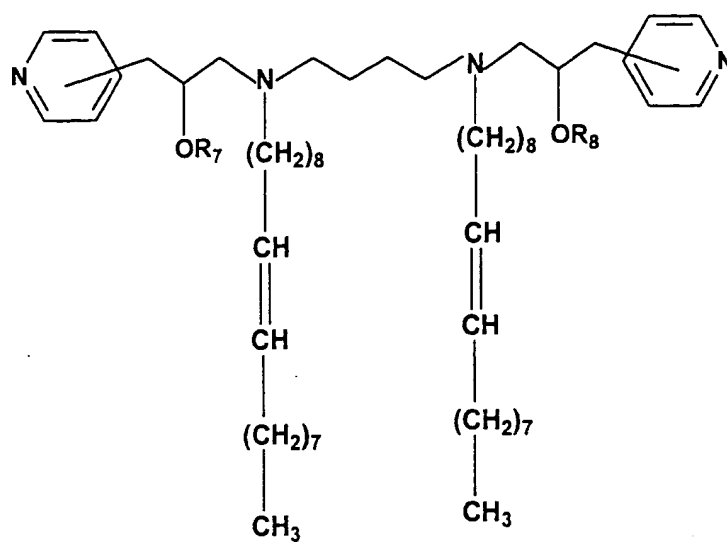


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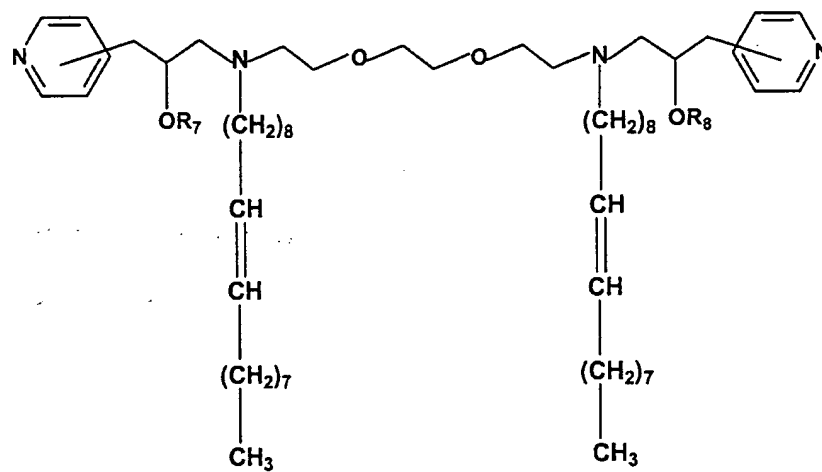


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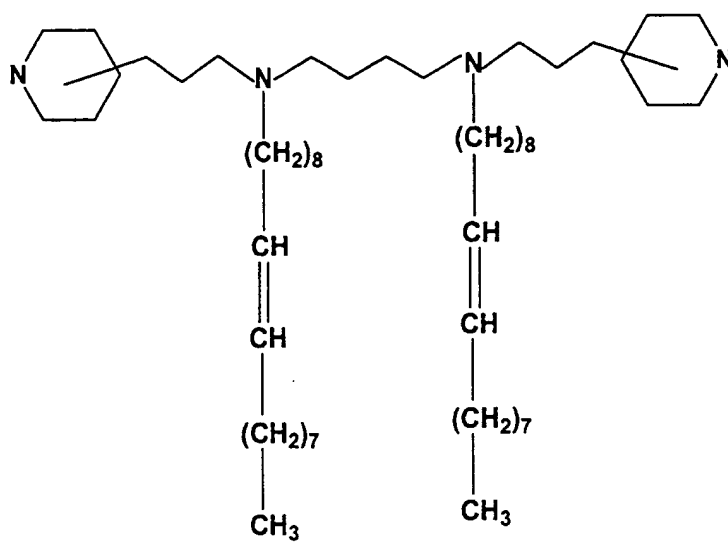


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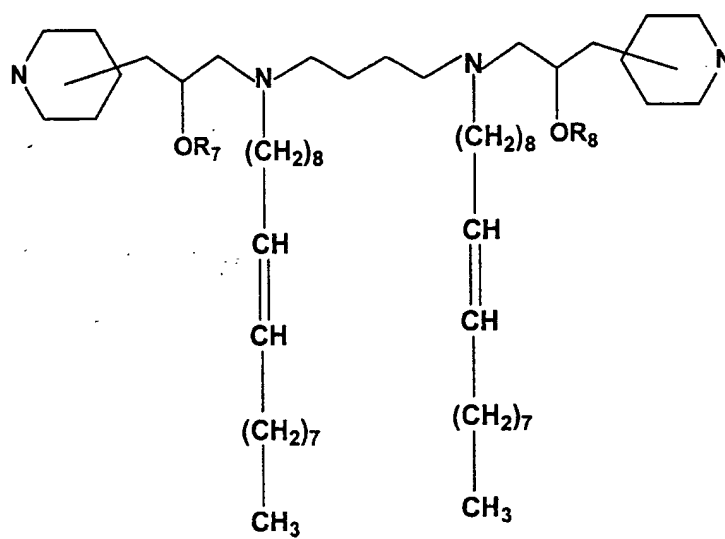


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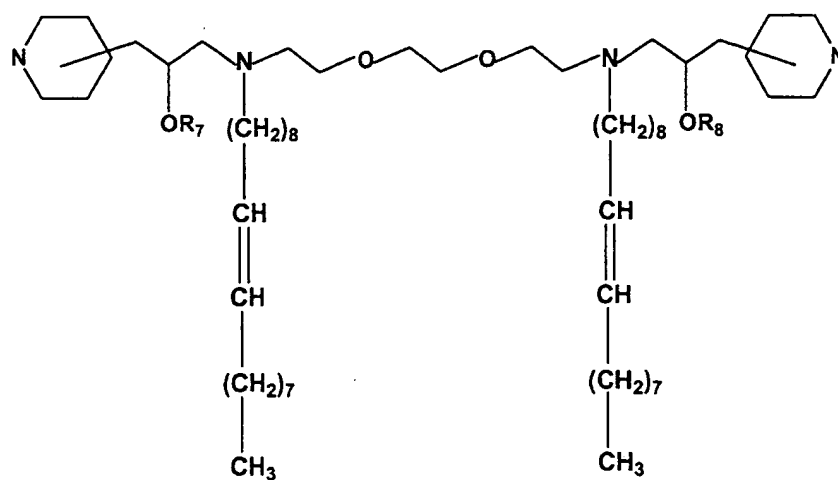


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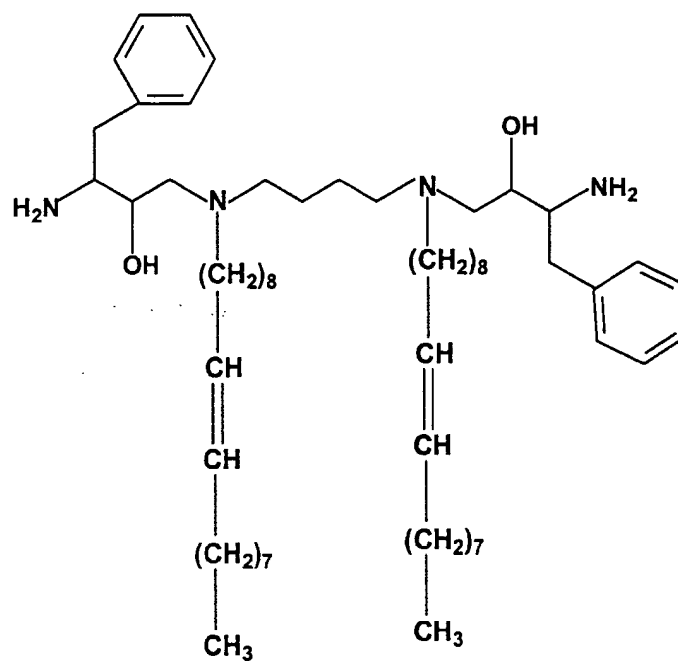


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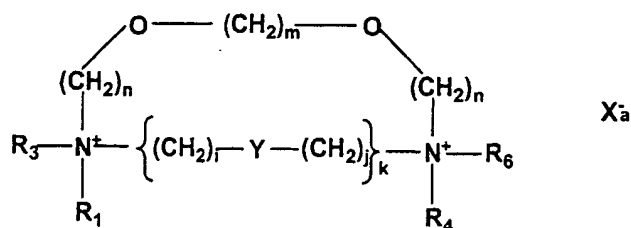
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5



Further, the compounds according to the present invention have the Formula (C):



5

wherein

Y is selected from the group consisting of CH₂, an ether, a polyether, an amide, a polyamide, an ester, a sulfide, a urea, a thiourea, a guanidyl, a carbamoyl, a carbonate, a phosphate, a sulfate, a sulfoxide, an imine, a carbonyl, and a secondary amino group and wherein Y is optionally substituted by -X₁-L'-X₂-Z or -Z;

R₁, R₃, R₄ and R₆, independently of one another, are selected from the group consisting of H, -(CH₂)_p-D-Z, an alkyl, an alkenyl, an aryl, and an alkyl or an alkyl ether optionally substituted by one or more of an alcohol, an aminoalcohol, an amine, an amide, an ether, a polyether, a polyamide, an ester, a mercaptan, an alkylthio, a urea, a thiourea, a guanidyl, or a carbamoyl group, and at least one of R₁, R₃, R₄ and R₆ is a straight chain or branched, cyclic, alkyl, alkenyl, alkynyl or aryl group, and R₁, R₃, R₄ and R₆ may optionally be covalently linked with each other or with Y, to form a cyclic moiety;

Z is selected from the group consisting of amine, spermiyl, carboxyspermiyl, guanidyl, spermidinyl, putricinyl, diaminoalkyl, pyridyl, piperidinyl, pyrrolidinyl, polyamine, amino acid, peptide, and protein;

X₁ and X₂, independently of one another, are selected from the group consisting of NH, O, S, alkylene, and arylene;

L' is selected from the group consisting of alkylene, alkenylene, alkynylene, arylene, alkylene ether, and polyether;

D is Q or a bond;

m and n are 0 or 1; and

i, j, k, l and p are integers independently selected from 1 to about 10.

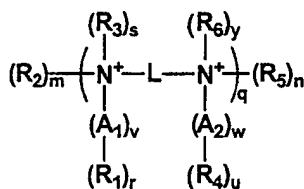
Preferably, Y is selected from the group consisting of CH₂, an ether, a polyether, an amide, a polyamide, an ester, a sulfide, a urea, a thiourea, a guanidyl, a carbamoyl, a carbonate, and a secondary amino group.

Preferably, R₁, R₃, R₄ and R₆, independently of one another, are selected from the group consisting of H, -(CH₂)_p-Z, an alkyl, an alkenyl, an aryl, and an alkyl or an alkyl ether optionally substituted by one or more of an alcohol, an aminoalcohol, an amine, an amide, an ether, a polyether, a polyamide, an ester, a mercaptan, a urea, a thiourea, a guanidyl, or a carbamoyl group, and at least one of R₁, R₃, R₄ and R₆ is a straight chain or branched, cyclic, alkyl, alkenyl, alkenyl or aryl group, and R₁, R₃, R₄ and R₆ may be covalently linked with each other, to form a cyclic moiety.

Preferably, at least one of R₁ and R₄ is straight chain or branched, cyclic, alkyl, alkenyl, alkynyl or aryl group having from about 2 to 100, preferably 4 to 75, more preferably 6 to 64, more preferably 8 to 50, more preferably 8 to 40, more preferably 8 to 30, more preferably 6 to 30, more preferably 4 to 30, and most preferably 8 to about 24 carbon atoms.

Preferably, Y is selected from the group consisting of CH₂, O, S and NH.

The compounds and polycations of the present invention have the following Formula (D):



wherein

L is C, CH, (CH₂)_i, or {(CH₂)_i-Y-(CH₂)_j}_k, wherein Y is selected from the group consisting of CH₂, an ether, a polyether, an amide, a polyamide, an ester, a sulfide, a urea, a thiourea, a guanidyl, a carbamoyl, a carbonate, a phosphate, a sulfate, a sulfoxide, an imine, a carbonyl, and a secondary amino group and wherein Y is optionally substituted by -X₁-L'-X₂-Z or -Z;

R₁ - R₆, independently of one another, are selected from the group consisting of H, -(CH₂)_p-D-Z, an alkyl, an alkenyl, an aryl, and an alkyl or an alkyl ether optionally

substituted by one or more of an alcohol, an aminoalcohol, an amine, an amide, an ether, a polyether, a polyamide, an ester, a mercaptan, an alkylthio, a urea, a thiourea, a guanidyl, or a carbamoyl group, and wherein at least one of R_1 , R_3 , R_4 and R_6 is a straight chain or branched, cyclic, alkyl, alkenyl, alkynyl or aryl group;

- 5 Z is selected from the group consisting of amine, spermyl, carboxyspermyl, guanidyl, spermidinyl, putricinyl, diaminoalkyl, pyridyl, piperidinyl, pyrrolidinyl, polyamine, amino acid, amino acid derivative, peptide, and protein;

X_1 and X_2 , independently of one another, are selected from the group consisting of NH, O, S, alkylene and arylene;

- 10 L' is selected from the group consisting of alkylene, alkenylene, alkynylene, arylene, alkylene ether, and polyether;

A_1 and A_2 , independently of one another, are selected from the group consisting of CH_2O , CH_2S , CH_2NH , $\text{C}(\text{O})$, $\text{C}(\text{NH})$, $\text{C}(\text{S})$ and $(\text{CH}_2)_i$;

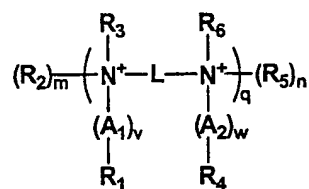
- 15 m, n, r, s, u, v, w and y are 0 or 1, with the proviso that when both m and n are 0 at least one of r, s, u and y is other than 0;

i, j, k, l, p and t are integers from 0 to about 100; and

q is an integer from 1 to about 1000.

Also, the compounds and the polycations of the present invention have the following Formula (E):

20



wherein

- 25 L is $(\text{CH}_2)_l$ or $\{(\text{CH}_2)_i - \text{Y} - (\text{CH}_2)_j\}_k$, wherein Y is selected from the group consisting of CH_2 , an ether, a polyether, an amide, a polyamide, an ester, a sulfide, a urea, a thiourea, a guanidyl, a carbamoyl, a carbonate, and a secondary amino group;

$R_1 - R_6$, independently of one another, are selected from the group consisting of H, $-(\text{CH}_2)_p - \text{Z}$, an alkyl, an alkenyl, an aryl, and an alkyl or an alkyl ether optionally substituted by one or more of an alcohol, an aminoalcohol, an amine, an amide, an ether,

a polyether, a polyamide, an ester, a mercaptan, a urea, a thiourea, a guanidyl, or a carbamoyl group, and at least one of R_1 , R_3 , R_4 and R_6 is a straight chain or branched, cyclic, alkyl, alkenyl, alkynyl or aryl group; Z , A_1 , A_2 , m , n , i , j , k , l , p , t and q are as defined above.

5 In the above formulae (D) and (E), R_1 - R_6 , Y , i , j , k , l , p , t and q are preferably as defined with reference to Formula (A).

It would be obvious for a skilled person that when Q is O or S, the number of substituents should be according their valency.

10 Certain of the compounds of the invention may be insufficiently soluble in physiological media to employ for delivery and transfection methods. Those of ordinary skill in the art will appreciate that there are a variety of techniques available in the art to enhance solubility of such compounds in aqueous media. Such methods are readily applicable without undue experimentation to the compounds described herein.

15 *Definitions*

Useful aryl groups are C_{6-100} aryl, preferably C_{6-75} aryl, more preferably C_{6-64} aryl, more preferably C_{6-50} aryl, more preferably C_{6-40} aryl, more preferably C_{6-30} aryl, most preferably C_{6-24} aryl. Typical C_{6-100} aryl groups include phenyl, naphthyl, phenanthryl, 20 anthracyl, indenyl, azulenyl, biphenyl, biphenylenyl, fluorenyl, pyrenyl, aceanthrenyl, cholantrenyl, acephenanthrenyl, violantherenyl, hexaphenyl, hexacenyl, trinaphtyl and pyranthyl groups.

Useful alkyl groups are straight chain or branched C_{2-100} alkyl groups, preferably C_{4-75} alkyl, more preferably C_{6-64} alkyl, more preferably C_{8-50} alkyl, more preferably C_{8-40} 25 alkyl, more preferably C_{8-30} alkyl, more preferably C_{6-30} alkyl, more preferably C_{4-30} alkyl, most preferably C_{8-24} alkyl. Typical C_{2-100} alkyl groups include ethyl, propyl, isopropyl, butyl, sec.-butyl, tert.-butyl, pentyl, hexyl, octyl, decyl, dodecyl, tetradecyl, hexadecyl, octadecyl, eicosyl, docosyl, tetracosyl, hexacosyl, octacosyl and triacontyl groups. Also contemplated is a trimethylene group substituted on two adjoining positions on any 30 benzene ring of the compounds of the invention.

Useful alkenyl groups are straight chain or branched C_{2-100} alkenyl, preferably C_{4-75} alkenyl, more preferably C_{6-64} alkenyl, more preferably C_{8-50} alkenyl, more preferably C_{8-40} alkenyl, more preferably C_{8-30} alkenyl, more preferably C_{6-30} alkenyl, more preferably C_{4-30} alkenyl, most preferably C_{8-24} alkenyl. Typical C_{2-100} alkenyl groups include ethenyl, propenyl, isopropenyl, butenyl, sec.-butenyl, hexenyl, octenyl, decenyl, dodecenyl, especially 9-dodecenyl, tetradecenyl, especially 9-tetradecenyl, hexadecenyl, especially 9-hexadecenyl, octadecenyl, especially 9-octadecenyl, eicosenyl, docosenyl, tetracosenyl, hexacosenyl, octacosenyl and triacontenyl.

Useful alkynyl groups are straight chain or branched C_{2-100} alkynyl, preferably C_{4-75} alkynyl, more preferably C_{6-64} alkynyl, more preferably C_{8-50} alkynyl, more preferably C_{8-40} alkynyl, more preferably C_{8-30} alkynyl, more preferably C_{6-30} alkynyl, more preferably C_{4-30} alkynyl, most preferably C_{8-24} alkynyl. Typical C_{2-100} alkynyl groups include ethynyl, propynyl, butynyl, -butynyl, hexynyl, octynyl, decynyl, dodecynyl, tetradecynyl, hexadecynyl, octadecynyl, eicosynyl, docosynyl, tetracosynyl, hexacosynyl, octacosynyl and triacontynyl groups.

Typical alkyl ether groups include any of the above-mentioned C_{2-100} alkyl groups having an ether group.

An ether group is $-O-$.

Typical polyether groups include the $-(CHR^{14}-CH_2-O)_t-$, wherein R^{14} is H or a C_{1-4} alkyl group and t is an integer as defined above, preferably t is 2 to 5.

For the purposes of the invention an amide group is an organic radical having $-NHC(O)-$ as a functional group. Typical amide groups include alkyl amides, alkenyl amides, alkynyl amides, and aryl amides, wherein alkyl, alkenyl, alkynyl and aryl are as defined above.

Typically polyamide groups include organic radicals having two or more amide groups as defined above.

Typically an ester group is an organic radical having $-C(O)-O-$ as a functional group. Typical ester groups include $R^{14}-C(O)-O-R^{15}$, wherein R^{14} and R^{15} are alkylene, alkenylene, alkynylene and arylene groups as defined above.

Typically urea groups are organic radicals having $-NH-C(O)-NH-$ as a functional group. Typical urea groups include $R^{14}NH-C(O)-NHR^{14}$,

$R^{14}NH-C(O)-NHR^{15}$, $R^{14}R^{15}N-C(O)-NR^{14}R^{15}$ wherein R^{14} and R^{15} are alkylene, alkenylene, alkynylene and arylene groups as defined above.

Typically thiourea groups are organic radicals having urea group as defined above wherein the oxygen in the urea group is substituted by sulfur.

- 5 Typically guanidyl groups are organic radicals having $-NH-C(NH)-NH-$ as a functional group. Typical guanidyl groups include $R^{14}NH-C(NH)-NHR^{14}$, $R^{14}NH-C(NH)-NHR^{15}$ and $R^{14}R^{15}N-C(NH)-NR^{14}R^{15}$ wherein R^{14} and R^{15} are alkylene, alkenylene, alkynylene and arylene groups as defined above.

A carbamoyl group is $-NH-C(O)-O-$.

- 10 Typically carbonate groups include organic radicals containing a CO_3^{2-} radical, i.e., $-O-C(O)-O-$.

A phosphate group is a PO_4^{3-} radical.

A sulfate group is a SO_4^{2-} radical.

A sulfoxide group is $-S(O)-$.

- 15 An imine group is $-C(N)-$.

A carbonyl group is $-C(O)-$.

A secondary amino group is $-NH-$.

- Typically aminoalcohol groups are organic radicals having both a secondary amino group as defined above and a hydroxyl group. Typical aminoalcohols include
20 aminoethanol, aminopropanol and aminobutanol.

The definition "D is a bond" means that when D is not Q there is a single bond between $(CH_2)_p$ and Z.

- Biologically Active Substance* refers to any molecule or mixture or complex of molecules that exerts a biological effect *in vitro* and/or *in vivo*, including
25 pharmaceuticals, drugs, proteins, peptides, polypeptides, hormones, vitamins, steroids, polyanions, nucleosides, nucleotides, nucleic acids (e.g. DNA or RNA), nucleotides, polynucleotides, etc.

Cationic Lipids refers to any cationic lipids which may be used for transfection, including but not limited to, DOSPA, DOTMA, DMRIE, DOTAP, DOGS and TM-TPS.

- 30 *Cell* refers to eukaryotic cells of any type and from any source. Types of eukaryotic cells include epithelial, fibroblastic, neuronal, hematopoietic cells and the like

from primary cells, tumor cells or immortalized cell lines. Sources of such cells include any animal such as human, canine, mouse, hamster, cat, bovine, porcine, monkey, ape, sheep, fish, insect, fungus and any plant including crop plants, ornamentals and trees.

Delivery is used to denote a process by which a desired compound is transferred
5 to a target cell such that the desired compound is ultimately located inside the target cell or in, or on, the target cell membrane. In many uses of the compounds of the invention, the desired compound is not readily taken up by the target cell and delivery via lipid aggregates is a means for getting the desired compound into the cell. In certain uses, especially under *in vivo* conditions, delivery to a specific target cell type is preferable and
10 can be facilitated by compounds of the invention.

Drug refers to any therapeutic or prophylactic agent other than food which is used in the prevention, diagnosis, alleviation, treatment, or cure of disease in man or animal.

Kit refers to transfection or protein expression kits which include one or more of the compounds of the present invention or mixtures thereof. Such kits may comprise a
15 carrying means being compartmentalized to receive in close confinement one or more container means such as vials, test tubes and the like. Each of such container means comprises components or a mixture of components needed to perform transfection. Such kits may include one or more components selected from nucleic acids (preferably one or more vectors), cells, one or more compounds of the present invention, lipid-aggregate
20 forming compounds, transfection enhancers, biologically active substances, etc.

Lipid Aggregate is a generic term which includes liposomes of all types both unilamellar and multilamellar as well as micelles and more amorphous aggregates of cationic lipids or lipids mixed with amphiphatic lipids such as phospholipids and steroids.

25 *Lipid Aggregate-forming Compounds* refers to neutral compounds or lipids such as DOPE, DOPC and cholesterol, etc.

Target Cell refers to any cell to which a desired compound is delivered, using a lipid aggregate as carrier for the desired compound.

Transfection is used herein to mean the delivery of nucleic acid, protein or other
30 macromolecule to a target cell, such that the nucleic acid, protein or other macromolecule is expressed or has a biological function in the cell. The term "expressible nucleic acid"

includes both DNA and RNA without regard to molecular weight, and the term "expression" means any manifestation of the functional presence of the nucleic acid within the cell including, without limitation, both transient expression and stable expression. Functional aspects include inhibition of expression by oligonucleotides or protein delivery.

Transfection Enhancers refers generally to molecules and substances such as proteins, peptides, growth factors and the like that enhance cell-targeting, uptake, internalization, nuclear targeting and expression. Such molecules and substances include ligands such as insulin, transferrin, fibronectin that target the cell surface; peptides that target cellular integrin receptors; and other compounds such as Plus Reagent (available from Life Technologies, Inc., Rockville, Maryland). Examples of transfection enhancers may be found in U.S. Patent No. 5,736,392 and U.S. application Serial No. 09/039,780 filed March 16, 1998.

The invention will be further clarified by the following examples, which are intended to be purely exemplary of the invention. The polycationic lipids were prepared by following the general reaction schemes described below.

Example 1

Synthesis of N',N'-dioleoyl-diaminobutane (I)

A solution of 1,4-diaminobutane (4.28 g, 48.6 mmol) and triethylamine (20.4 ml, 146 mmol) in 10 mL of dry methylene chloride was slowly added to a solution of oleoyl chloride (30.0 g, 99.7 mmol) in 300 ml of anhydrous methylene chloride in an ice bath at 0°C. The reaction mixture was stirred vigorously with a mechanical stirrer. After the addition was complete, the ice bath was removed and the mixture was stirred at room temperature for 2.5 days. TLC analysis confirmed that the reaction had gone to completion and the product had precipitated. The excess oleoyl chloride was removed by filtration. The precipitate was washed twice with 50 ml of methylene chloride. The mother liquor was concentrated and more product precipitated. This precipitate was filtered and combined with the previous precipitate. The resulting solid was vacuum

dried for 4 hours. A total of 27.0 g of a white solid of the desired product, N¹,N⁴-dioleoyl-diaminobutane, was obtained.

Synthesis of N¹,N⁴-dioleoyl-diaminobutane (II)

5

Lithium aluminum hydride (8.62 g, 95%, 216 mmol) was carefully added to a suspension of N¹,N⁴-dioleoyl-diaminobutane (27.0 g, 43.8 mmol) in 400 ml of anhydrous diethyl ether at 0°C. After addition, the ice bath was removed. The reaction mixture was warmed slowly to room temperature and then heated gently to reflux with an appropriate
10 condensing device and stirred for 16 hours. The reaction mixture was then cooled and quenched carefully at 0°C with 70 mL of a 1 N sodium hydroxide solution. Another 500 mL of diethyl ether was added and the mixture was stirred at room temperature for additional 2 hours. The top ether layer turned clear gradually and then separated. The aqueous layer was extracted three times with 100 mL of diethyl ether each. The
15 combined ether solution was concentrated, and dried on high vacuum overnight. Total of 17.0 g of oily colorless N¹,N⁴-dioleoyl-diaminobutane was obtained.

Synthesis of N¹,N⁴-dioleoyl-N¹,N⁴-di-[2-hydroxy-3-(N-phthalamido)propyl]-diamino-butane (III)

20

Diisopropylethylamine (11.1 mL, 63.7 mmol) was added to a suspension of N¹,N⁴-dioleoyl-diaminobutane (15.5 g, 26.3 mmol) and N-(2,3-epoxypropyl)-phthalimide (15.6 g, 76.8 mmol) in 110 mL of dry N,N-dimethylformamide. After purging with nitrogen, the reaction mixture was sealed in a round-bottom flask and heated to around
25 90°C for 24 hours. N,N-dimethylformamide and diisopropylethylamine were removed and a yellow oil was obtained. This crude material was recrystallized from ethanol. A total of 18.6 g of a white solid, N¹,N⁴-dioleoyl-N¹,N⁴-di-[2-hydroxy-3-(N-phthalamido)propyl]-diamino-butane was obtained.

Synthesis of N¹,N⁴-dioleyl-N¹,N⁴-di-[2-hydroxy-3-(N-aminopropyl)]-diaminobutane (IV) (hereinafter referred to as DHDOS)

Hydrazine (4.0 mL, 80% aq., 103 mmol) was added to a suspension of N¹,N⁴-dioleyl-N¹,N⁴-di-[2-hydroxy-3-(N-phthalamido)propyl]-diaminobutane (17.0 g, 17.1 mmol) in 250 mL of dry ethanol at room temperature. With an appropriate condensing device, the reaction mixture was heated to a reflux, at which point the suspension turned into a clear solution. The oil bath was set to 85°C. After 45 minutes a white solid precipitated from the solution. The reaction mixture was stirred at reflux for 4 hours before being cooled to -20°C. The white solid settled down to the bottom. The top clear ethanol solution was decanted. The residue was washed twice with cold ethanol. The combined ethanol solution was concentrated and dried overnight over vacuum. 12.4 g of oily N¹,N⁴-dioleyl-N¹,N⁴-di-[2-hydroxy-3-(N-aminopropyl)]-diaminobutane was obtained.

15

The following compounds were synthesized by the above method using the corresponding diamine and a long chain acyl chloride:

- N¹,N⁴-dimyristyl-N¹,N⁴-di-[2-hydroxy-3-(N-aminopropyl)]-diaminobutane;
- N¹,N⁴-dipalmityl-N¹,N⁴-di-[2-hydroxy-3-(N-aminopropyl)]-diaminobutane;
- N¹,N⁴-dipalmitoyl-N¹,N⁴-di-[2-hydroxy-3-(N-aminopropyl)]-diaminobutane;
- N¹,N⁴-distearyl-N¹,N⁴-di-[2-hydroxy-3-(N-aminopropyl)]-diaminobutane;
- N¹,N⁴-dilauryl-N¹,N⁴-di-[2-hydroxy-3-(N-aminopropyl)]-diaminobutane;
- N¹,N²-dimyristyl-N¹,N²-di-[2-hydroxy-3-(N-aminopropyl)]-diaminoethane;
- N¹,N²-dipalmityl-N¹,N²-di-[2-hydroxy-3-(N-aminopropyl)]-diaminoethane;
- N¹,N²-dipalmitoyl-N¹,N²-di-[2-hydroxy-3-(N-aminopropyl)]-diaminoethane;
- N¹,N²-distearyl-N¹,N²-di-[2-hydroxy-3-(N-aminopropyl)]-diaminoethane;
- N¹,N²-dilauryl-N¹,N²-di-[2-hydroxy-3-(N-aminopropyl)]-diaminoethane;
- N¹,N²-dioleyl-N¹,N²-di-[2-hydroxy-3-(N-aminopropyl)]-diaminoethane;
- N¹,N⁹-dimyristyl-N¹,N⁹-di-[2-hydroxy-3-(N-aminopropyl)]-Jeffamine;
- N¹,N⁹-dipalmityl-N¹,N⁹-di-[2-hydroxy-3-(N-aminopropyl)]-Jeffamine;

N^1, N^9 -dipalmitoyl- N^1, N^9 -di-[2-hydroxy-3-(N-aminopropyl)-Jeffamine;
 N^1, N^9 -distearyl- N^1, N^9 -di-[2-hydroxy-3-(N-aminopropyl)-Jeffamine;
 N^1, N^9 -dilauryl- N^1, N^9 -di-[2-hydroxy-3-(N-aminopropyl)-Jeffamine;
 N^1, N^9 -dioleoyl- N^1, N^9 -di-[2-hydroxy-3-(N-aminopropyl)-Jeffamine.

5

Synthesis of N^1, N^4 -dioleoyl - N^1, N^4 -di-[2-hydroxy-3-(N-carboxamidine)-aminopropyl]-diaminobutane (V)

1H-pyrazole-1-carboxamidine hydrochloride (45 mg, 0.31 mmol) was added to
 10 a solution of N^1, N^4 -dioleoyl- N^1, N^4 -di-[2-hydroxy-3-(N-aminopropyl)]-diamino-butane
 (115 mg, 0.156 mmol) in 1 mL of dry N,N-dimethylformamide. The salt was not very
 soluble in dimethylformamide (DMF). However, the mixture turned clear after
 diisopropylethylamine (55 μ l, 0.31 mmol) was added. The mixture was stirred under
 nitrogen at room temperature for 18 hours. After removal of solvent, the crude material
 15 was loaded on a C-18 reverse phase flash column, and eluted with 20% H_2O in MeOH
 to 10% H_2O in MeOH. The pure fractions were collected and concentrated. An 81 mg
 colorless oily N^1, N^4 -dioleoyl- N^1, N^4 -di-[2-hydroxy-3-(N-carboxamidine)aminopropyl]-
 diaminobutane was obtained, which was converted to its TFA and HCL salts.

20

Synthesis of N^1, N^4 -dioleoyl- N^1, N^4 -di-[2-hydroxy-3-[N-($N^I, N^{II}, N^{III}, N^{IV}$ -butoxycarbonyl-spermine carboxamido)]aminopropyl]diaminobutane (VI)

Diisopropylcarbodiimide (5.32 mL, 34.0 mmol) was added dropwise to a solution
 of Boc-spermine acid (21.7 g, 33.5 mmol) and N-hydroxysuccinimide (NHS) (3.91 g,
 25 34.0 mmol) in mixed solvents (100 mL of DMF and 100 mL of CH_2Cl_2) at room
 temperature. After stirring for 2.5 hours, a solution of N^1, N^4 -dioleoyl- N^1, N^4 -di-[2-
 hydroxy-3-(N-aminopropyl)] diaminobutane (10 g, 13.6 mmol) in 40 mL of methylene
 chloride and DMF was added. The mixture was stirred for another 5 hours before
 quenching with 200 mL of a 2.5% sodium bicarbonate solution. An additional 300 mL
 30 of methylene chloride was added. The aqueous solution was extracted with 120 mL of
 methylene chloride three times. The combined organic solution was washed with water

twice and dried over anhydrous magnesium sulfate. After concentration, a pale yellow oil was obtained. The crude material was purified with silica gel, eluting with 2% MeOH in CH₂Cl₂ to 5% MeOH in CH₂Cl₂. A total of 13.1 g of white solid N^I,N⁴-dioleyl-N^I,N⁴-di-{2-hydroxy-3-[N-(N^I,N^{II},N^{III},N^{IV}-butoxycarbonylspermine carboxamido)]aminopropyl}di-aminobutane was obtained.

Synthesis of N^I,N⁴-dioleyl - N^I,N⁴-di-[2-hydroxy-3-(N-spermine carboxamido)-aminopropyl] -diaminobutane (VII)

10 100 mL of a solution of 4.0 M hydrogen chloride in 1,4-dioxane was added to a solution of N^I,N⁴-dioleyl-N^I,N⁴-di-{2-hydroxy-3-[N-(N^I,N^{II},N^{III},N^{IV}-butoxycarbonylspermine carboxamido)]aminopropyl}diaminobutane (11.8 g, 5.92 mmol) in 100 mL of 1,4-dioxane at room temperature. The reaction mixture turned cloudy 10 minutes after addition of the acid. After 2.5 hours of stirring at room temperature, the excess acid and
15 solvent was removed. The residue was dried for at least 5 hours over vacuum before being loaded on a C-18 reverse phase flash column. The column was eluted starting with 25% H₂O in MeOH, then 20%, and then 17%. Pure fractions were collected and concentrated. A 3.06 g colorless solid N^I,N⁴-dioleyl-N^I,N⁴-di-[2-hydroxy-3-(N-spermine carboxamido)-aminopropyl]-diaminobutane was obtained.

20

The following compounds were synthesized using the protocol described above starting with the requisite diamine and long chain acyl chloride:

N^I,N⁴-dimyristyl-N^I,N⁴-di-[2-hydroxy-3-(N-sperminecarboxamido)-aminopropyl]-diaminobutane;

25 N^I,N⁴-dipalmityl-N^I,N⁴-di-[2-hydroxy-3-(N-sperminecarboxamido)-aminopropyl]-diaminobutane;

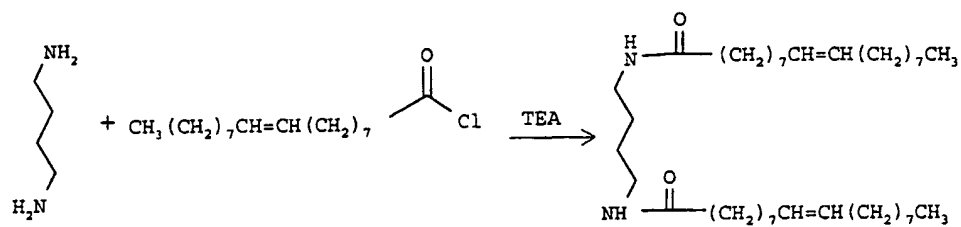
N^I,N⁴-dipalmitoyl-N^I,N⁴-di-[2-hydroxy-3-(N-sperminecarboxamido)-aminopropyl]-diaminobutane;

30 N^I,N⁴-distearyl-N^I,N⁴-di-[2-hydroxy-3-(N-sperminecarboxamido)-aminopropyl]-diaminobutane;

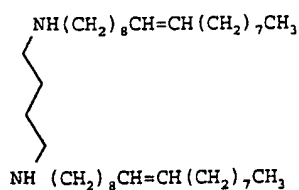
- N^1, N^4 -dilauryl- N^1, N^4 -di-[2-hydroxy-3-(N-sperminecarboxamido)-aminopropyl]-diaminobutane;
- N^1, N^9 -dimyristyl- N^1, N^9 -di-[2-hydroxy-3-(N-sperminecarboxamido)-aminopropyl]-Jeffamine;
- 5 N^1, N^9 -dipalmityl- N^1, N^9 -di-[2-hydroxy-3-(N-sperminecarboxamido)-aminopropyl]-Jeffamine;
- N^1, N^9 -dipalmitoyl- N^1, N^9 -di-[2-hydroxy-3-(N-sperminecarboxamido)-aminopropyl]-Jeffamine;
- 10 Jeffamine;
- N^1, N^9 -dilauryl- N^1, N^9 -di-[2-hydroxy-3-(N-sperminecarboxamido)-aminopropyl]-Jeffamine;
- N^1, N^9 -dioleyl- N^1, N^9 -di-[2-hydroxy-3-(N-sperminecarboxamido)-aminopropyl]-Jeffamine;
- 15 N^1, N^2 -dimyristyl- N^1, N^2 -di-[2-hydroxy-3-(N-sperminecarboxamido)-aminopropyl]-diaminoethane;
- N^1, N^2 -dipalmityl- N^1, N^2 -di-[2-hydroxy-3-(N-sperminecarboxamido)-aminopropyl]-diaminoethane;
- N^1, N^2 -dipalmitoyl- N^1, N^2 -di-[2-hydroxy-3-(N-sperminecarboxamido)-aminopropyl]-diaminoethane;
- 20 N^1, N^2 -distearyl- N^1, N^2 -di-[2-hydroxy-3-(N-sperminecarboxamido)-aminopropyl]-diaminoethane;
- N^1, N^2 -dilauryl- N^1, N^2 -di-[2-hydroxy-3-(N-sperminecarboxamido)-aminopropyl]-diaminoethane;
- 25 N^1, N^2 -dioleyl- N^1, N^2 -di-[2-hydroxy-3-(N-sperminecarboxamido)-aminopropyl]-diaminoethane.

-60-

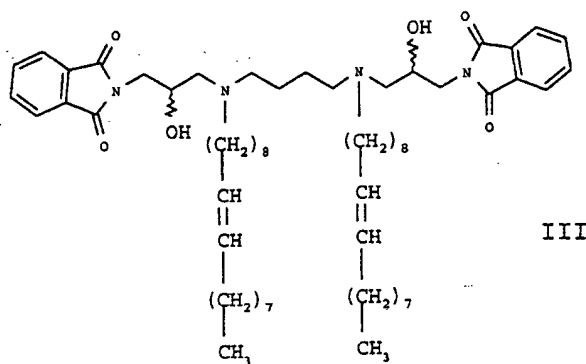
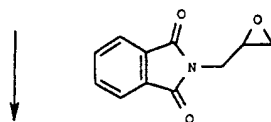
**Synthesis of dihydroxy-dioleoyl-disperminecarboxamido
spermine and analogs (Scheme 1)**



I



II



III

NCCCCN(CCCCNC(CO)CCCN)C(O)CCCN
 $\xrightarrow{\text{NH}_2\text{NH}_2}$

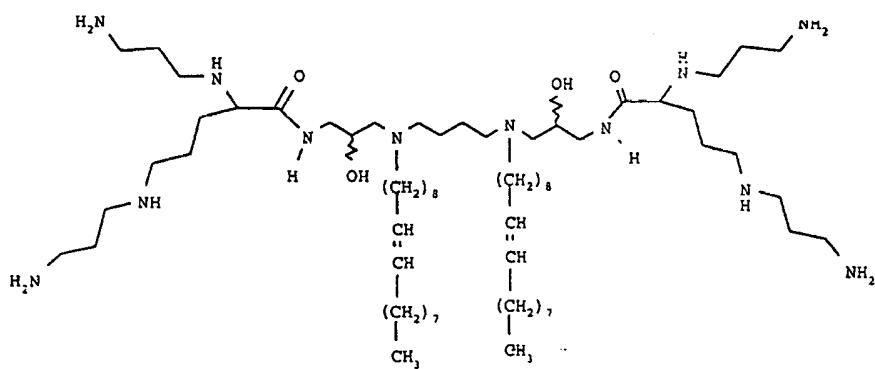
1) Boc-spermic acid (VI)

2) acid

The diagram shows a chemical reaction where a long-chain molecule reacts with hydrazine (NH_2NH_2) to form a bis-aldimine product labeled V. The starting material has two terminal amino groups and two internal hydroxyl groups. The product V has two terminal aldimine groups ($\text{HN}=\text{CH}-\text{NH}_2$) and two internal hydroxyl groups. A reagent box contains a pyridine ring and a guanidine group ($\text{HN}=\text{CH}-\text{NH}_2 \cdot \text{HCl}$). Below the main reaction, there are two numbered steps: 1) Boc-spermic acid (VI) and 2) acid.

V

- 1) Boc-spermic acid (VI)
- 2) acid



VII

Example 2

Synthesis of N¹,N⁴-dioleyl - N¹,N⁴-di-3-cyanopropyl-diaminobutane (VIII)

5

Acrylonitrile (0.43 mL, 6.53 mmol) was added dropwise to a solution of N¹,N⁴-dioleyl-diaminobutane (1.8 g, 3.06 mmol) in 20 mL of ethanol at room temperature. The mixture was stirred for 30 hours. All starting materials were converted to product as confirmed by TLC analysis. The crude material was purified using flash chromatography with a silica gel (1% MeOH in CH₂Cl₂). A clear oil was obtained at high yield.

10

Synthesis of N¹,N⁴-dioleyl-N¹,N⁴-di-3-(aminopropyl)-diaminobutane (IX)

15

A solution of LAH (9.2 mL, 1 M in ether, 9.2 mmol) was slowly added to a solution of N¹,N⁴-dioleyl-N¹,N⁴-di-3-cyanopropyl-diaminobutane (2.12 g, 3.05 mmol) in 15 mL of anhydrous diethyl ether at 0°C. After addition, the mixture was stirred at room temperature for 20 hours. All starting material was consumed. The reaction mixture was quenched with a 1 N NaOH solution at 0°C. After stirring 2 hours at room temperature, the mixture was extracted with diethyl ether three times. The combined ether solutions were concentrated and dried over vacuum for three hours. An oily N¹,N⁴-dioleyl-N¹,N⁴-di-3-(aminopropyl)diaminobutane was obtained at high yield.

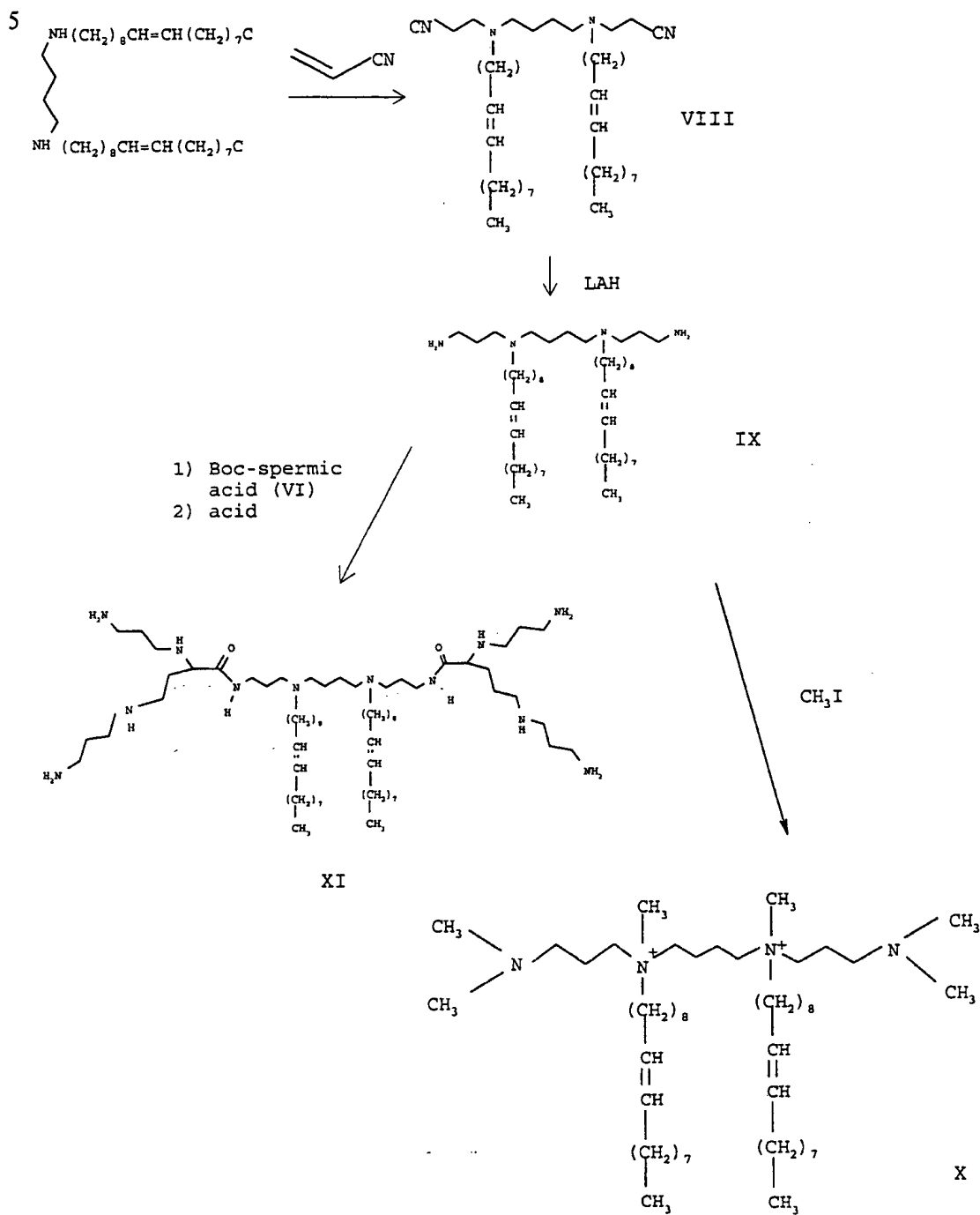
20

Synthesis of N¹,N⁴-dioleyl-N¹,N⁴-di-[3-(N-spermine carboxamido)-aminopropyl]-diaminobutane (XI)

25

The procedure for making N¹,N⁴-dioleyl-N¹,N⁴-di-[2-hydroxy-3-(N-spermine carboxamido)-aminopropyl]-diaminobutane described above was followed.

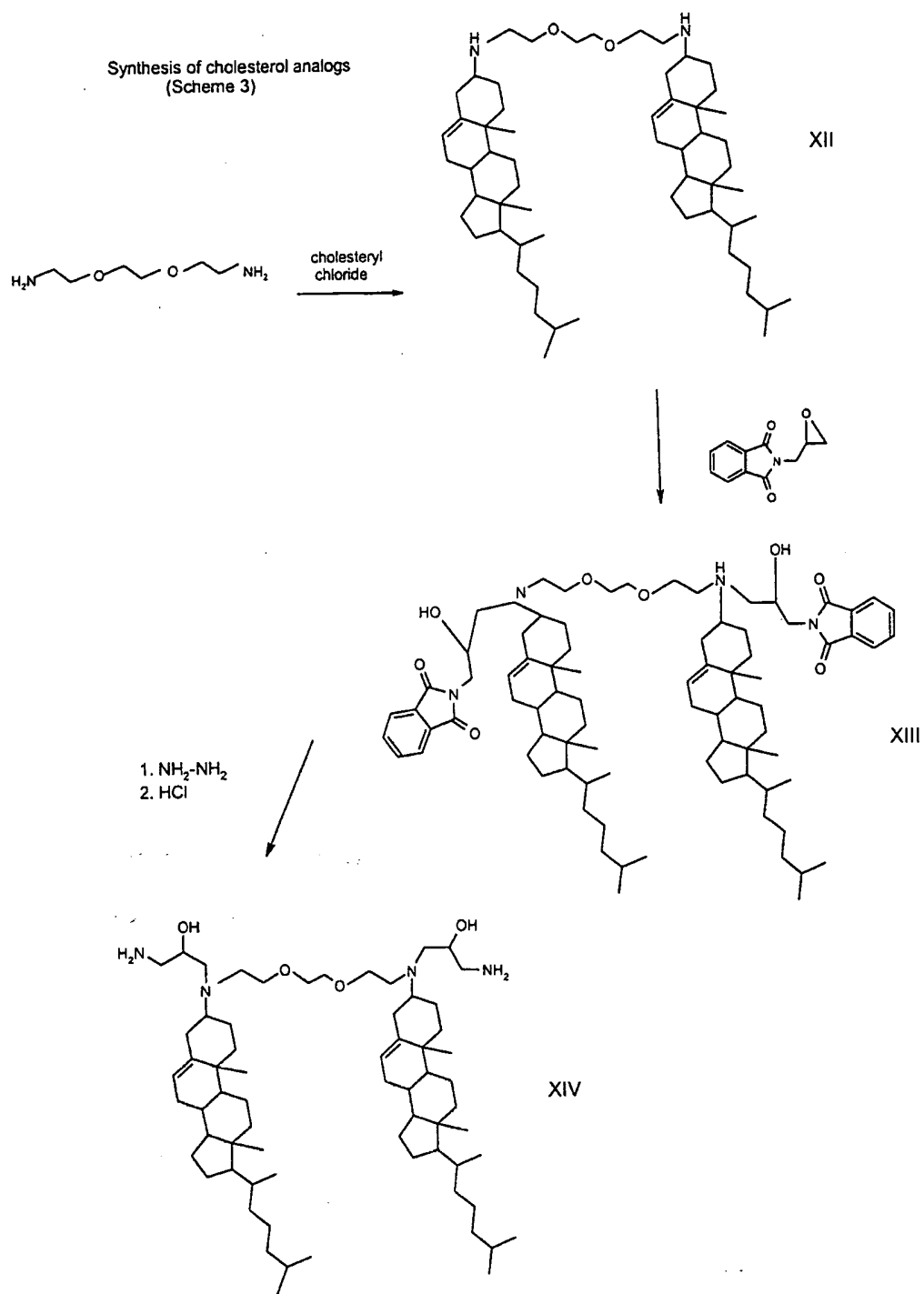
*Synthesis of dioleyol-dispermincarboxamido spermine
and analogs (Scheme 2)*



*Example 3**Synthesis of cholesterol analogs*

- 5 The cholesterol analogs can be synthesized by using the scheme given below (Scheme 3). Jefamine is alkylated with cholesteryl chloride to provide the dicholesteryl jefamine analog (XII). Further alkylation with the epoxide phthalamide (XIII) and deblocking with hydrazine gives the compound of the invention (XIV).

-65-

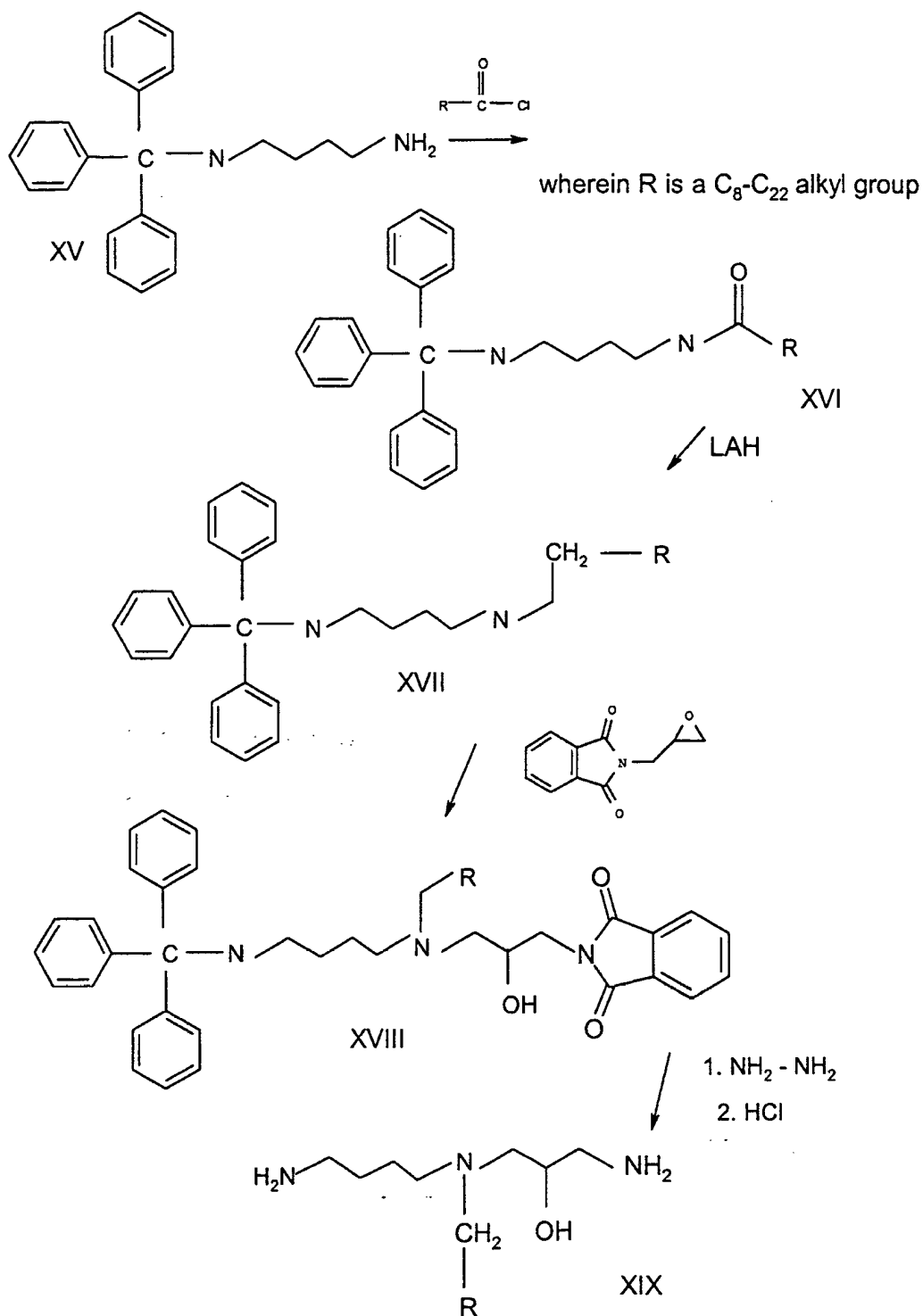
Synthesis of cholesterol analogs
(Scheme 3)

Example 4

Synthesis of monoalkyl analogs

- 5 When monoalkyl analogs are desired, the above Scheme 1 can be modified such that one of the amines in the starting material is protected before the acylation step. Thus, tritylprotected diaminobutane (XV) is acylated with alkanoyl chloride (e.g., oleoyl chloride) followed with LAH reduction to obtain compound XVIII. The amine is then alkylated with the desired phthalamide epoxide to obtain compound XVIII. Removing
10 the phthalamide using hydrazine renders the desired amine XIX. (See Scheme 4).

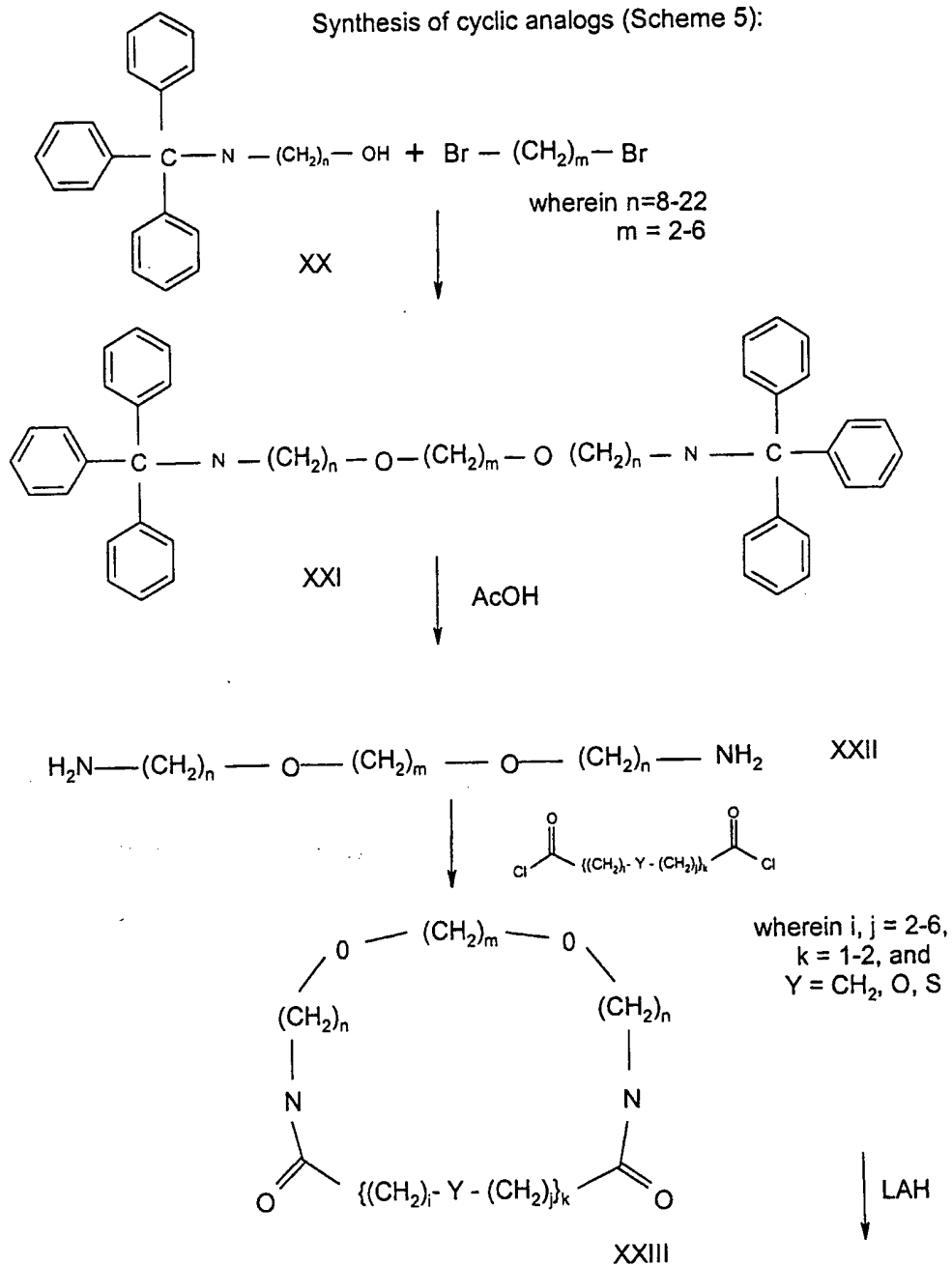
Synthesis of monoalkyl analogs (Scheme 4):

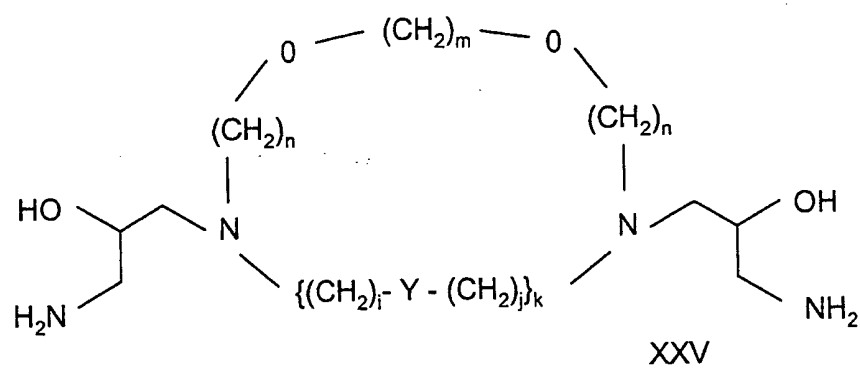
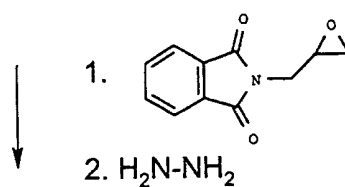
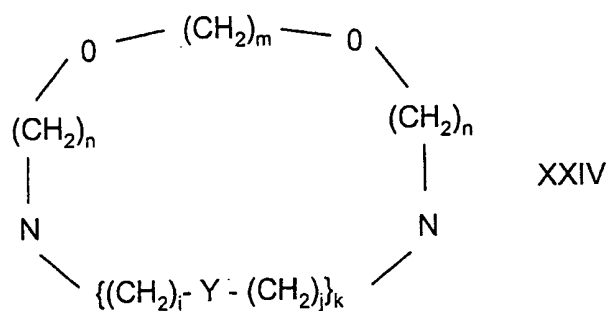


*Example 5**Synthesis of cyclic analogs*

- 5 The following scheme (Scheme 5) can be used to make the cyclic analogs. Trityl protected amino alcohol (XX) with the desired chain is alkylated using dibromoalkyl (e.g., dibromobutane). The trityl is removed from the desired dimer (XXI) and acylated using diacyl chlorides (e.g., succinyl chloride). The amide (XXIII) is then reduced with LAH and alkylated using the desired phthalamide epoxide. Removal of the phthalamide
- 10 gives the desired compound of the invention.

Synthesis of cyclic analogs (Scheme 5):





Example 6

Synthesis of polymeric analogs

5 Polymeric analogs of the present invention can be synthesized by using polymeric amines such as PEI as starting material or dendrimeric polyamines. For example, PEI can be acylated with alkylol chloride (e.g., oleoyl chloride) and the acylated PEI can then be reduced with lithium aluminum hydride to obtain compounds of the invention.

10 Although the above methods exemplifies the synthesis of specific compounds, the reaction schemes provide a general method for preparing a variety of compounds according to the present invention. Those of ordinary skill in the art will appreciate that alternate methods and reagents other than those specifically detailed herein can be employed or readily adapted to produce compounds of the invention.

15 The compounds of the present invention can be used in the same manner as are prior art compounds such as DOTMA, DOTAP, DOGS, DOSPA and the like. Methods for incorporating such cationic lipids into lipid aggregates are well-known in the art. Representative methods are disclosed by Felgner, et al., *supra*; Eppstein et al. *supra*; Behr et al. *supra*; Bangham, A. et al. (1965) M. Mol. Biol. 23:238-252; Olson, F. et al. (1979) Biochim. Biophys. Acta 557:9-23; Szoka, F. et al. (1978) Proc. Natl. Acad. Sci. USA 75:4194-4198; Mayhew, E. et al. (1984) Biochim. Biophys. Acta 775:169-175; Kim, S. et al. (1983) Biochim. Biophys. Acta 728: 339-348; and Fukunaga, M. et al. (1984) Endocrinol. 115:757-761. Techniques for preparing lipid aggregates of appropriate size for use as delivery vehicles include sonication and freeze-thaw plus extrusion as perhaps
20 the most commonly used. See, e.g., Mayer, L. et al. (1986) Biochim. Biophys. Acta 858:161-168. Microfluidization is used when consistently small (50-200 nm) and relatively uniform aggregates are desired (Mayhew, E., *supra*). Aggregates ranging from about 50 nm to about 200 nm diameter are preferred; however, both larger and smaller sized aggregates are functional.

25 Methods of transfection and delivery of other compounds are well-known in the art. The compounds and compositions of the present invention yield lipid aggregates that

can be used in the same processes used for other known transfection agents.

It will be readily apparent to those of ordinary skill in the art that a number of general parameters are important for optimal efficiency of transfection or delivery. These parameters include, for example, the cationic lipid concentration, the concentration of
5 compound to be delivered, the number of cells transfected, the medium employed for delivery, the length of time the cells are incubated with the polyanion-lipid complex, and the relative amounts of cationic and non-cationic lipid. It may be necessary to optimize these parameters for each particular cell type. Such optimization is routine employing the guidance provided herein and knowledge generally available to the art.

10 It will also be apparent to those of ordinary skill in the art that alternative methods, reagents, procedures and techniques other than those specifically detailed herein can be employed or readily adapted to produce the liposomal precursors and transfection compositions of this invention. Such alternative methods, reagents, procedures and techniques are within the spirit and scope of this invention.

15 The use of representative compounds of the invention are further detailed by reference to the following examples. All abbreviations used herein are standard abbreviations in the art. Specific procedures not described in detail are either referenced or well-known in the art.

20

Example 7

This example compares transfection of HEK-293 (human embryonic kidney-derived cell line), COS-7 (SV40 transformed monkey cell line), CHO-K1 (Chinese Hamster Ovary-derived cell line), and HeLa (Human cervical carcinoma-derived cell
25 line) cells with the β -galactosidase reporter plasmid DNA pCMV \cdot SPORT- β -gal (LifeTechnologies, Rockville, MD) using commercially available cationic lipid transfection reagents and the compounds of the present invention.

The cells were plated the day before transfection in 24-well tissue culture plates in a total volume of 0.4ml DMEM (Dulbecco's Modified Eagle's Medium, Life
30 Technologies, Rockville, MD) culture medium containing a 1% non-essential amino acid (NEAA) solution (LifeTechnologies), and 10% FBS. For the HEK-293 and COS-7 cells,

the tissue culture plates were pre-coated with Poly-L-Lysine to enhance cell attachment.

The next day, DNA-transfection reagent complexes were prepared as follows:

The cationic lipid reagents and DNA were diluted separately into 25 μ l aliquots of serum-free DMEM, containing 1% NEAA. For LipofectAMINE PLUS, 7-14 μ l of PLUS reagent was added to the DNA, mixed, and incubated for 15 minutes at room temperature. The diluted DNA was combined with the diluted lipid and incubated at room temperature for at least 15 minutes to allow the DNA and the lipid to form complexes. Following this incubation the complexes were added directly into the culture medium dropwise and mixed by rocking the culture plate back and forth. The cells were further incubated at 37°C for a total of 24 hours to allow expression of the lacZ transgene encoded by the reporter plasmid, pCMV•SPORT- β -gal. At 24 hours post-transfection, the growth medium and transfection complexes were removed from the wells, and the cells in each well were rinsed briefly with 1ml of D-PBS (Dulbecco's PBS, Life Technologies, Rockville, MD). The cells in each well were lysed by the addition of 0.15 to 2.0 ml of 0.1% Tris, pH 8.0, containing 0.1 M Triton X-100. The plates were frozen at -80°C for a minimum of 2 hours, and thawed at room temperature or 37°C. The thawed cell lysates were cleared by centrifugation and the supernatants were assayed for β -gal activity using the enzymatic substrate ONPG. The concentration of total protein in cell lysates was also determined using a Bradford assay (Bio-Rad Laboratories, Hercules CA). β -gal activity in transfected cell extracts was calculated against a standard curve and expressed as ng β -gal per surface area of tissue culture plate (ng/cm²) to reflect total activity per transfection, or as ng β -gal per μ g of total protein (ng/ μ g) to reflect specific activity.

HEK-293 (Figure 1), COS-7 (Figure 2), CHO-K1 (Figure 3), and HeLa (Figure 4) cells were transfected with 0.4 or 0.8 μ g of pCMV•SPORT- β -gal DNA and 0.2 to 4.0 μ l of transfection reagent. The transfection reagents tested were DHDOS (IV) formulated at 2mg/ml with the neutral co-lipid, cholesterol (at a ratio of 1:15 (M/M) DHDOS to cholesterol); DHDOS formulated at 2mg/ml with the neutral co-lipid DOPE (dioleoyl phosphatidyl ethanolamine) (at a ratio of 1:1 (M/M) DHDOS to DOPE); LipofectAMINE PLUS (Life Technologies, Rockville MD); and FuGENE™-6 (Boehringer Mannheim, Germany). DHDOS formulations were tested in the range of 0.2 to 1.5 μ l;

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LipofectAMINE PLUS and FuGENE-6 were tested in the range of 0.2 to 4.0 μ l. FuGENE-6 was used according to the manufacturer's recommended protocol. DHDOS and LipofectAMINE PLUS were used according to the above protocol. The data presented in the Figures are expressed as total activity (ng/cm²) to better compare total expression from the transfected DNA. Only data with 0.8 μ g of DNA is shown, since similar results were obtained with 0.4 and 0.8 μ g of DNA.

Example 8

Primary, passaged, normal human fibroblasts (NHFs) were plated in 96-well plates at a density of 1.6×10^4 cells per well and transfected the following day. Cells in each well were transfected with 40 ng pCMV•SPORT- β -gal DNA and 0.1 or 0.2 μ l lipid. The DNA and lipid were diluted separately into 10 μ l of DMEM. The DNA was either used alone or pre-mixed with PLUS, insulin, transferrin, or an integrin-targeting peptide prior to complexing with the lipid. After 15 minutes of complexing, the DNA-lipid was added to cells. Cells were assayed for β -gal activity as described above.

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ACTIVITY (ng/ β gal/cm²)

5	<u>LIPID</u>	ACTIVITY (ng/ β gal/cm ²)				DNA and INTEGRIN- TARGETING PEPTIDE**
		<u>DNA</u>	<u>DNA and PLUS*</u>	<u>DNA and INSULIN</u>	<u>DNA and TRANSFERRIN</u>	
	LipofectAMINE	10.36	28.6	ND	17.4	ND
10	Compound of Formula X 1:1.5 DOPE 1 mg/ml	ND	37.8	ND	ND	40.9
15	Compound of Formula VII 1:1 DOPE 2 mg/ml	29.4	637.9	195.7	21.7	587.9

20 ND = no detectable activity

* PLUS Reagent is available from Life Technologies, Inc., Rockville, Maryland.

** Reference: S.L. HART, et al (1998), Human Gene Therapy, 9:575-585.

25

The results show that these cationic lipid formulations can deliver DNA molecules alone, but also that delivery, and ultimately gene expression, may be enhanced when the lipids are used in conjunction with peptides or proteins that bind DNA and/or act as ligands for cell surface receptors, or otherwise enhance cellular and/or nuclear uptake.

30

Having now fully described the present invention in some detail by way of illustration and examples for purposes of clarity of understanding, it will be obvious to one of ordinary skill in the art that the same can be performed by modifying or changing the invention within a wide and equivalent range of conditions, formulations and other parameters without affecting the scope of the invention or any specific embodiment thereof, and that such modifications or changes are intended to be encompassed within the scope of the appended claims.

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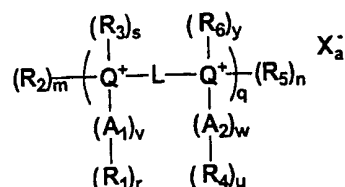
All publications, patents and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains, and are herein incorporated by reference to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be

5 incorporated by reference.

What Is Claimed Is:

1. A compound having the formula:

5



wherein

Q is selected from the group consisting of N, O and S;

- 10 L is C, CH, $(CH_2)_i$, or $\{(CH_2)_i - Y - (CH_2)_j\}_k$, wherein Y is selected from the group consisting of CH_2 , an ether, a polyether, an amide, a polyamide, an ester, a sulfide, a urea, a thiourea, a guanidyl, a carbamoyl, a carbonate, a phosphate, a sulfate, a sulfoxide, an imine, a carbonyl, and a secondary amino group and wherein Y is optionally substituted by $-X_1-L'-X_2-Z$ or $-Z$;

- 15 $R_1 - R_6$, independently of one another, are selected from the group consisting of H, $-(CH_2)_p-D-Z$, an alkyl, an alkenyl, an aryl, and an alkyl or alkyl ether optionally substituted by one or more of an alcohol, an aminoalcohol, an amine, an amide, an ether, a polyether, a polyamide, an ester, a mercaptan, an alkylthio, a urea, a thiourea, a guanidyl, or a carbamoyl group, and wherein at least one of R_1 , R_3 , R_4 and R_6 is a straight chain or branched, cyclic, alkyl, alkenyl, alkynyl or aryl group having from about 6 to about 64 carbon atoms; and R_1 and R_4 or R_3 and R_6 may optionally be covalently linked
- 20 with each other, with Y or with L when L is C or CH to form a cyclic moiety;

Z is selected from the group consisting of amine, spermiyl, carboxyspermiyl, guanidyl, spermidinyl, putricinyl, diaminoalkyl, pyridyl, piperidinyl, pyrrolidinyl, polyamine, amino acid, peptide, and protein;

- 25 X_1 and X_2 , independently of one another, are selected from the group consisting of NH, O, S, alkylene, and arylene;

L' is selected from the group consisting of alkyl, alkenylene, alkynylene, arylene, alkylene ether, and polyether;

D is Q or a bond;

A_1 and A_2 , independently of one another, are selected from the group consisting of CH_2O , CH_2S , CH_2NH , $\text{C}(\text{O})$, $\text{C}(\text{NH})$, $\text{C}(\text{S})$ and $(\text{CH}_2)_i$;

X is a physiologically acceptable anion;

m , n , r , s , u , v , w and y are 0 or 1, with the proviso that when both m and n are 0

5 at least one of r , s , u and y is other than 0;

i , j , k , l , p and t are integers from 0 to about 100;

q is an integer from 1 to about 1000; and

a is the number of positive charge divided by the valence of the anion.

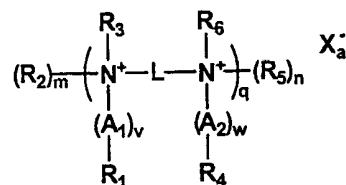
10 2. The compound as claimed in claim 1, wherein at least one of R_1 , R_3 , R_4 and R_6 is a straight chain or branched, cyclic, alkyl, alkenyl, alkynyl or aryl having from about 8 to about 24 carbon atoms.

3. The compound as claimed in claim 1, wherein the alkyl ether optionally
15 substituted by one or more alcohol groups comprises a carbohydrate.

4. The compound as claimed in claim 3, wherein the carbohydrate is selected from the group consisting of galactose, fructose, glucose, maltose, sucrose, cellobiose, lactose, mannose, glucopyranose, mannopyranose and galactopyranose.

20

5. A compound as claimed in claim 1, wherein said compound has the formula:



25 wherein

L is $(\text{CH}_2)_i$ or $\{(\text{CH}_2)_i - Y - (\text{CH}_2)_j\}_k$, wherein Y is selected from the group consisting of CH_2 , an ether, a polyether, an amide, a polyamide, an ester, a sulfide, a urea, a thiourea, a guanidyl, a carbamoyl, a carbonate, and a secondary amino group;

$R_1 - R_6$, independently of one another, are selected from the group consisting of H, $-(CH_2)_p-Z$, an alkyl, an alkenyl, an aryl, and an alkyl or alkyl ether optionally substituted by one or more of an alcohol, an aminoalcohol, an amine, an amide, an ether, a polyether, a polyamide, an ester, a mercaptan, a urea, a thiourea, a guanidyl, or a carbamoyl group, and at least one of R_1 , R_3 , R_4 and R_6 is a straight chain or branched, cyclic, alkyl, alkenyl, alkynyl or aryl group having from about 6 to about 64 carbon atoms; and R_1 and R_4 or R_3 and R_6 may optionally be covalently linked with each other to form a cyclic moiety;

Z is selected from the group consisting of amine, spermiyl, carboxyspermiyl, guanidyl, spermidinyl, putricinyl, diaminoalkyl, pyridyl, piperidinyl, pyrrolidinyl, polyamine, amino acid, peptide, and protein;

A_1 and A_2 , independently of one another, are selected from the group consisting of CH_2O , CH_2S , CH_2NH , $C(O)$, $C(NH)$, $C(S)$ and $(CH_2)_i$;

X is a physiologically acceptable anion;

m, n, v and w are 0 or 1;

i, j, k, l, p and t are integers from 1 to about 100;

q is an integer from 1 to about 1000; and

a is the number of positive charge divided by the valence of the anion, wherein when m and n are 0, then a is 0.

20

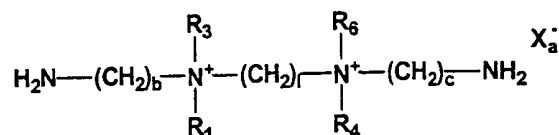
6. The compound as claimed in claim 5, wherein at least one of R_1 , R_3 , R_4 and R_6 is a straight chain or branched, cyclic, alkyl, alkenyl, alkynyl or aryl having from about 8 to about 24 carbon atoms.

25 7. The compound as claimed in claim 5, wherein the alkyl ether optionally substituted by one or more alcohol groups comprises a carbohydrate.

8. The compound as claimed in claim 7, wherein the carbohydrate is selected from the group consisting of galactose, fructose, glucose, maltose, sucrose, cellobiose, lactose, mannose, glucopyranose, mannopyranose and galactopyranose.

30

9. The compound as claimed in claim 5, wherein said compound has the formula:



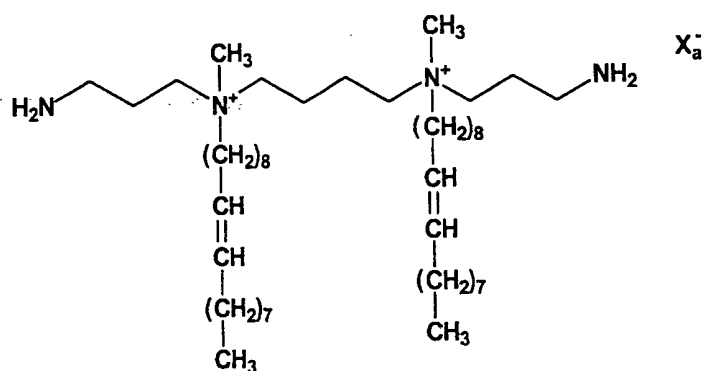
- 5 wherein

- R_1 , R_3 , R_4 and R_6 , independently of one another, are selected from the group consisting of H and a C_1 - C_8 alkyl, alkenyl, aryl, and alkyl optionally substituted by one or more of an alcohol, an amine, an amide, an ether, a polyether, a polyamide, an ester, a mercaptan, a urea, a thiourea, a guanidyl, or a carbamoyl group, and at least one of R_1 , R_3 , R_4 and R_6 is a straight chain or branched, cyclic, alkyl, alkenyl, alkynyl or aryl group having from about 8 to about 24 carbon atoms; and

1, b and c are integers independently selected from 1 to about 4.

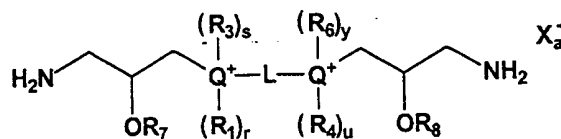
10. The compound as claimed in claim 9, which is:

15



11. The compound as claimed in claim 1, wherein said compound has the formula:

20



wherein

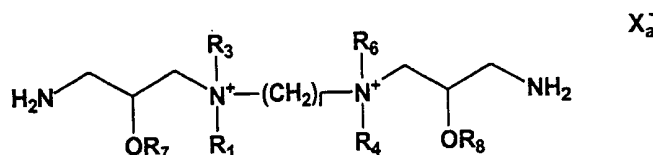
Q and L are as defined in claim 1;

R_1 , R_3 , R_4 and R_6 , independently of one another, are selected from the group consisting of H and a C_1 - C_8 alkyl, alkenyl, aryl, and alkyl optionally substituted by one or more of an alcohol, an amine, an amide, an ether, a polyether, a polyamide, an ester, a mercaptan, a urea, a thiourea, a guanidyl, or a carbamoyl group;

r , s , u and y are 0 or 1; and

R_7 and R_8 are independently H or a carbohydrate.

12. The compound as claimed in claim 11, wherein said compound has the formula:



wherein

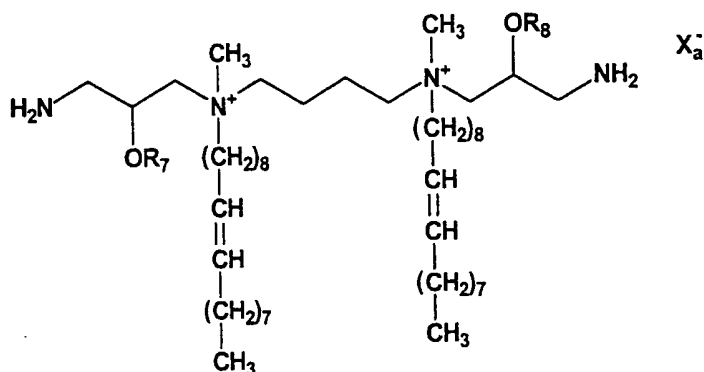
R_1 , R_3 , R_4 and R_6 , independently of one another, are selected from the group consisting of H and a C_1 - C_8 alkyl, alkenyl, aryl, and alkyl optionally substituted by one or more of an alcohol, an amine, an amide, an ether, a polyether, a polyamide, an ester, a mercaptan, a urea, a thiourea, a guanidyl, or a carbamoyl group, and at least one of R_1 , R_3 , R_4 and R_6 is a straight chain or branched, cyclic, alkyl, alkenyl, alkenyl or aryl group having from about 8 to about 24 carbon atoms;

R_7 and R_8 are independently H or a carbohydrate; and

l is an integer from 1 to about 4.

13. The compound as claimed in claim 12, which is:

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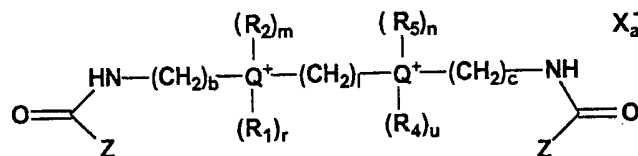
wherein R_7 and R_8 are independently H or a carbohydrate.

5

14. The compound as claimed in claim 13, wherein R_7 and R_8 are H.

15. The compound as claimed in claim 1, wherein said compound has the formula:

10



wherein

Q is as defined in claim 1;

15 R_1 , R_2 , R_4 and R_5 , independently of one another, are selected from the group consisting of H and a C_1 - C_8 alkyl, alkenyl, aryl, and alkyl optionally substituted by one or more of an alcohol, an amine, an amide, an ether, a polyether, a polyamide, an ester, a mercaptan, a urea, a thiourea, a guanidyl, or a carbamoyl group;

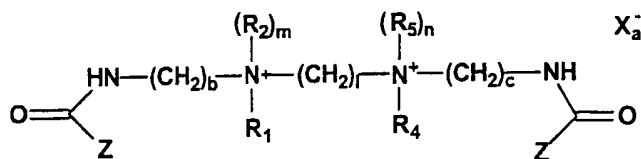
Z is selected from the group consisting of spermyl, spermidyl, amino acid, peptidyl, diaminoalkyl, and polyamine;

20

m, n, r and u are 0 or 1; and

l, b and c are integers independently selected from 1 to about 4.

16. The compound as claimed in claim 5, wherein said compound has the formula:



5 wherein

R_1 , R_2 , R_4 and R_5 , independently of one another, are selected from the group consisting of H and a C_1 - C_8 alkyl, alkenyl, aryl, and alkyl optionally substituted by one or more of an alcohol, an amine, an amide, an ether, a polyether, a polyamide, an ester,

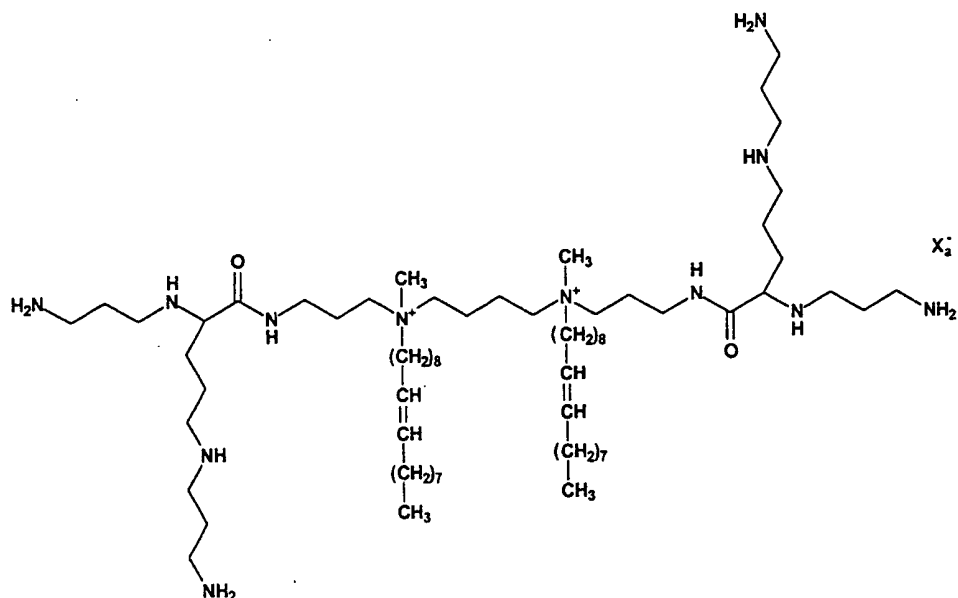
10 R_2 , R_4 and R_5 is a straight chain or branched, cyclic, alkyl, alkenyl, alkynyl or aryl group having from about 8 to about 24 carbon atoms;

Z is selected from the group consisting of spermiyl, spermidyl, amino acid, peptidyl, diaminoalkyl, and polyamine;

m and n are 0 or 1; and

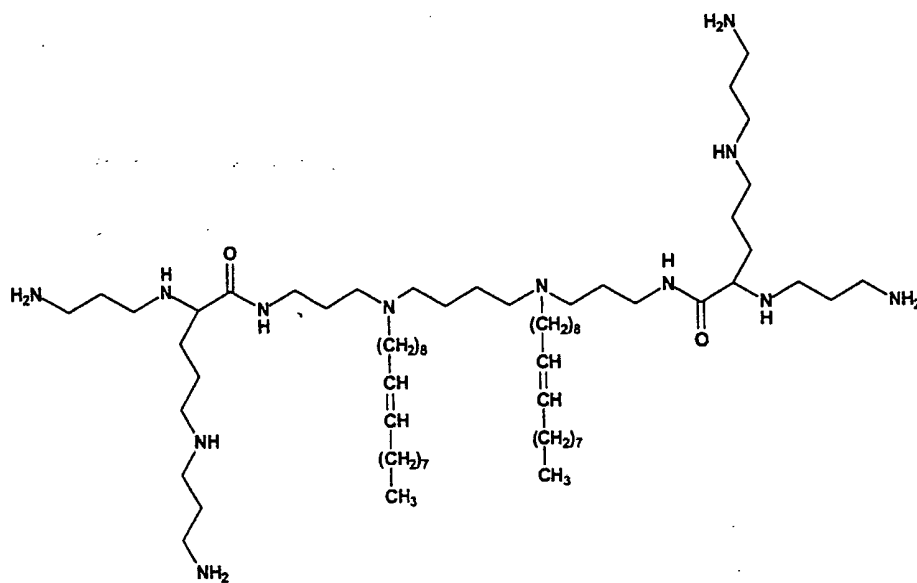
15 l , b and c are integers independently selected from 1 to about 4.

17. The compound as claimed in claim 16, which is:

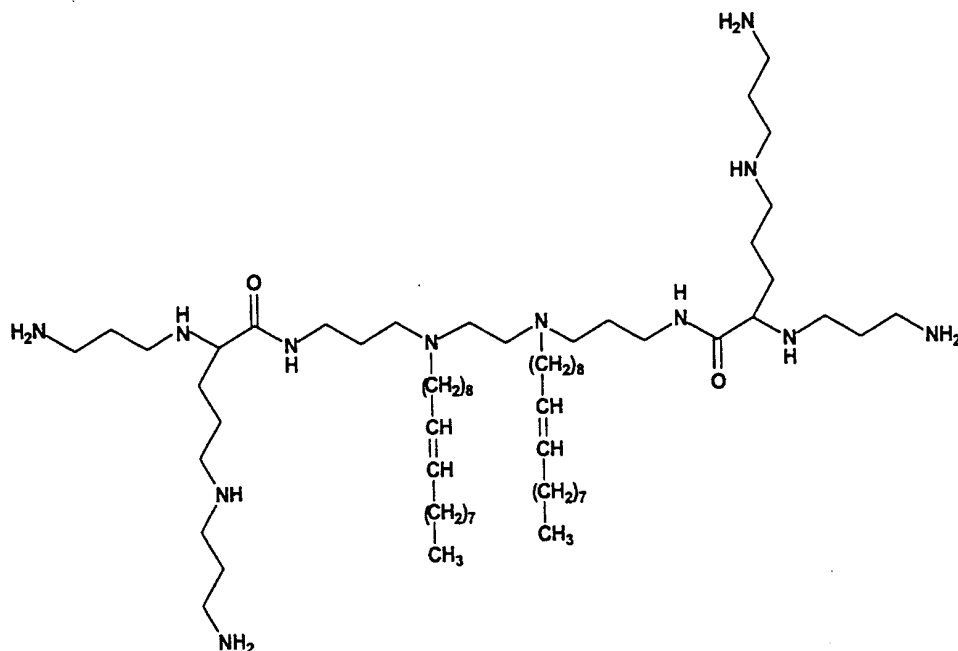


18. The compound as claimed in claim 16, which is:

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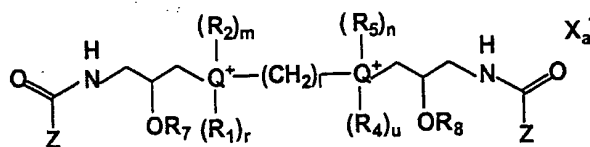


19. The compound as claimed in claim 16, which is:



5

20. The compound as claimed in claim 1, wherein said compound has the formula:



10

wherein

Q , R_1 , R_4 , m , n , r and u are as defined in claim 1;

R_2 and R_5 , independently of one another, are selected from the group consisting of H and a C_1 - C_8 alkyl, alkenyl, aryl, and alkyl optionally substituted by one or more of an alcohol, an amine, an amide, an ether, a polyether, a polyamide, an ester, a mercaptan, a urea, a thiourea, a guanidyl, or a carbamoyl group;

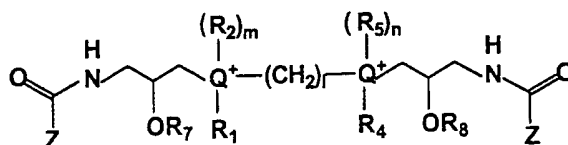
Z is selected from the group consisting of spermyl, spermidyl, amino acid, peptidyl, diaminoalkyl, and polyamine;

R_7 and R_8 are independently H or a carbohydrate; and

l is an integer from 1 to about 4.

21. The compound as claimed in claim 20, wherein said compound has the formula:

5



wherein

Q as defined in claim 1;

10 at least one of R_1 and R_4 is a straight chain or branched, cyclic, alkyl, alkenyl, alkynyl or aryl group having from about 8 to about 24 carbon atoms;

R_2 and R_5 , independently of one another, are selected from the group consisting of H and a C_1 - C_8 alkyl, alkenyl, aryl, and alkyl optionally substituted by one or more of an alcohol, an amine, an amide, an ether, a polyether, a polyamide, an ester, a mercaptan, 15 a urea, a thiourea, a guanidyl, or a carbamoyl group;

Z is selected from the group consisting of spermyl, spermidyl, amino acid, peptidyl, diaminoalkyl, and polyamine;

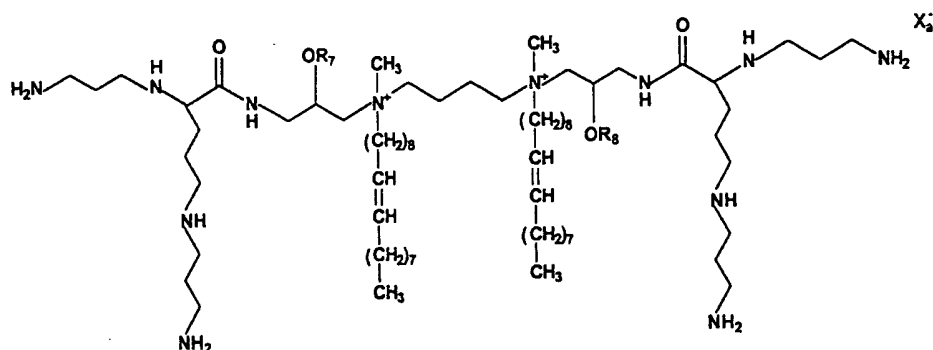
R_7 and R_8 are independently H or a carbohydrate; m and n are as defined in claim 1; and

20 l is an integer from 1 to about 4.

22. The compound as claimed in claim 21, wherein Q is N and R_7 and R_8 are H.

23. The compound as claimed in claim 21 which is:

25



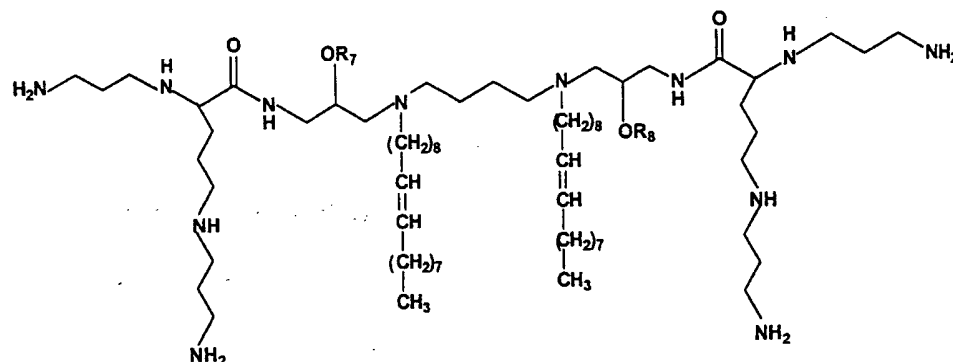
wherein R₇ and R₈ are independently H or a carbohydrate.

5

24. The compound according to claim 23, wherein R₇ and R₈ are H.

25. The compound as claimed in claim 21, which is:

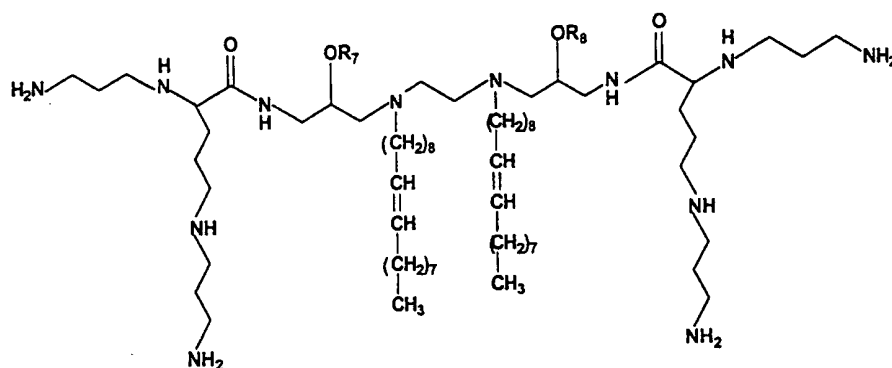
10



wherein R₇ and R₈ are independently H or a carbohydrate.

- 15 26. The compound as claimed in claim 25, wherein R₇ and R₈ are H.

27. The compound as claimed in claim 21, which is:



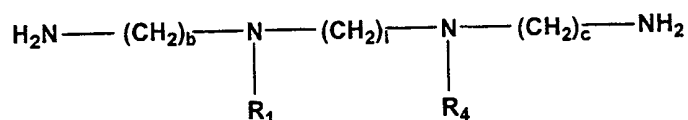
wherein R_7 and R_8 are independently H or a carbohydrate.

5

28. The compound as claimed in claim 27, wherein R_7 and R_8 are H.

29. The compound as claimed in claim 5, wherein said compound has the formula:

10



wherein

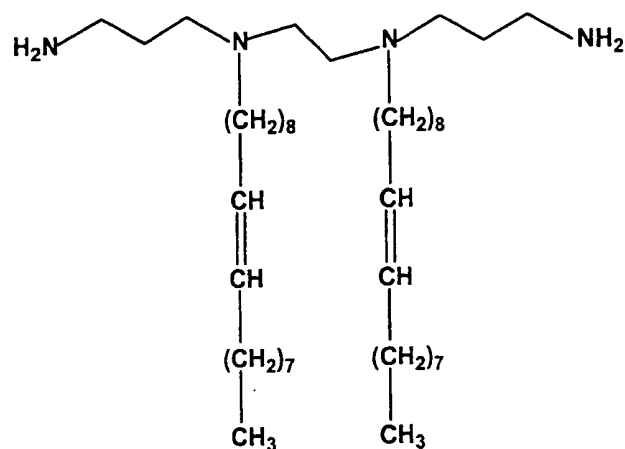
at least one of R_1 and R_4 is a straight chain or branched, cyclic, alkyl, alkenyl,

15 alkynyl or aryl group having from about 8 to about 24 carbon atoms; and

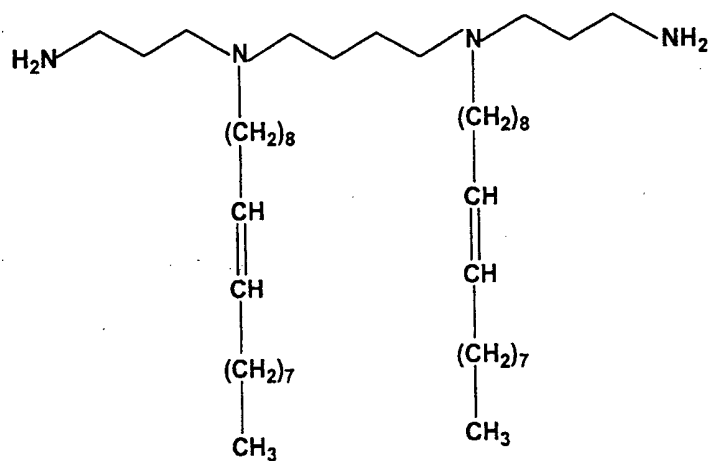
l, b and c are integers independently selected from 1 to about 4.

30. The compound as claimed in claim 29, which is:

-89-

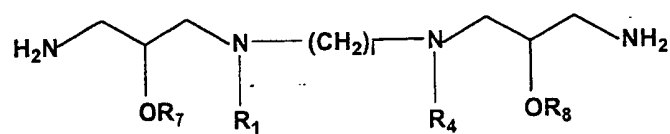


- 5 31. The compound as claimed in claim 29, which is:



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32. The compound as claimed in claim 5, wherein said compound has the formula:



15

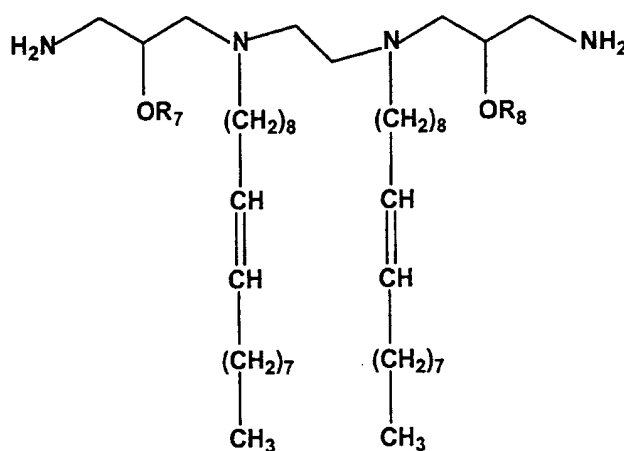
wherein

at least one of R_1 and R_4 is a straight chain or branched, cyclic, alkyl, alkenyl, alkynyl or aryl group having from about 8 to about 24 carbon atoms; R_7 and R_8 are independently hydrogen or a carbohydrate; and

5 1 is an integer from 1 to about 4.

33. The compound as claimed in claim 32, which is:

10



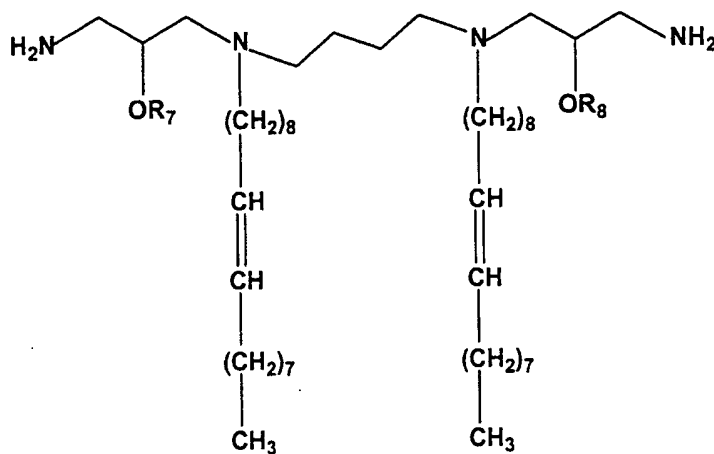
wherein R_7 and R_8 are independently H or a carbohydrate.

34. The compound as claimed in claim 33, wherein R_7 and R_8 are H.

15

35. The compound as claimed in claim 32, which is:

-91-



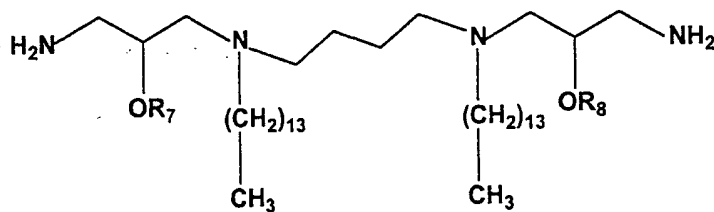
wherein R_7 and R_8 are independently H or a carbohydrate.

5

36. The compound as claimed in claim 35, wherein R_7 and R_8 are H.

37. The compound as claimed in claim 32, which is:

10



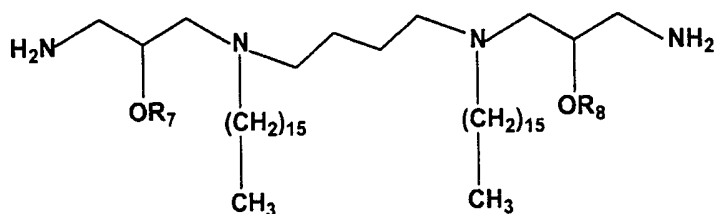
wherein R_7 and R_8 independently are H or a carbohydrate.

15

38. The compound as claimed in claim 37, wherein R_7 and R_8 are H.

39. The compound as claimed in claim 32, which is:

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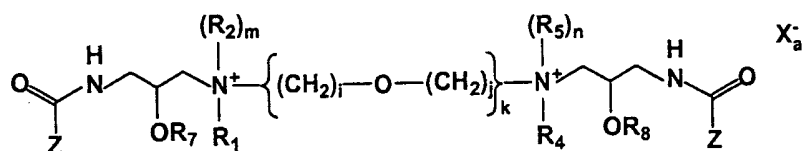
wherein R_7 and R_8 are H or a carbohydrate.

5

40. The compound as claimed in claim 39, wherein R_7 and R_8 are H.

41. The compound as claimed in claim 5, wherein said compound has the formula:

10



wherein

Z is as defined in claim 5;

15

at least one of R_1 and R_4 is a straight chain or branched, cyclic, alkyl, alkenyl, alkynyl or aryl group having from about 8 to about 24 carbon atoms;

R_2 and R_5 , independently of one another, are selected from the group consisting of H and a C_1 - C_8 alkyl, alkenyl, aryl, and alkyl optionally substituted by one or more of an alcohol, an amine, an amide, an ether, a polyether, a polyamide, an ester, a mercaptan,

20 a urea, a thiourea, a guanidyl, or a carbamoyl group;

R_7 and R_8 are independently H or a carbohydrate;

m and n are 0 or 1;

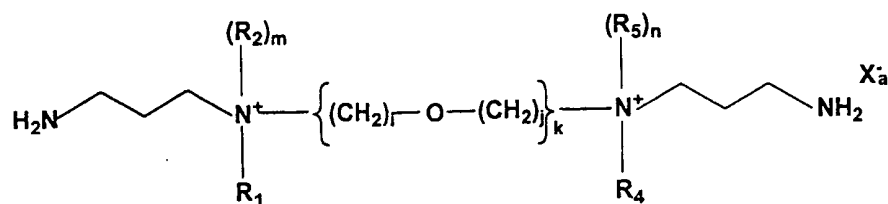
i and j are integers from about 2 to about 3; and

k is an integer from 1 to about 3.

25

42. The compound as claimed in claim 5, wherein said compound has the formula:

-93-



wherein

- 5 at least one of R_1 and R_4 is a straight chain or branched, cyclic, alkyl, alkenyl, alkynyl or aryl group having from about 8 to about 24 carbon atoms;

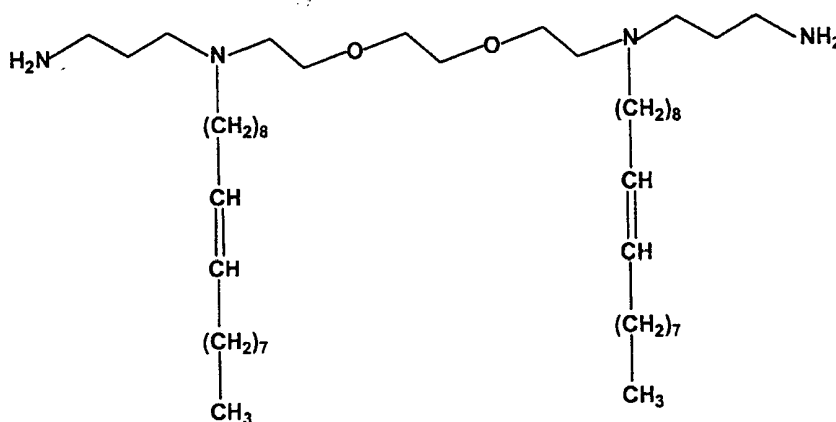
- R_2 and R_5 , independently of one another, are selected from the group consisting of H and a $\text{C}_1\text{-C}_8$ alkyl, alkenyl, aryl, and alkyl optionally substituted by one or more of an alcohol, an amine, an amide, an ether, a polyether, a polyamide, an ester, a mercaptan, 10 a urea, a thiourea, a guanidyl, or a carbamoyl group;

m and n are 0 or 1;

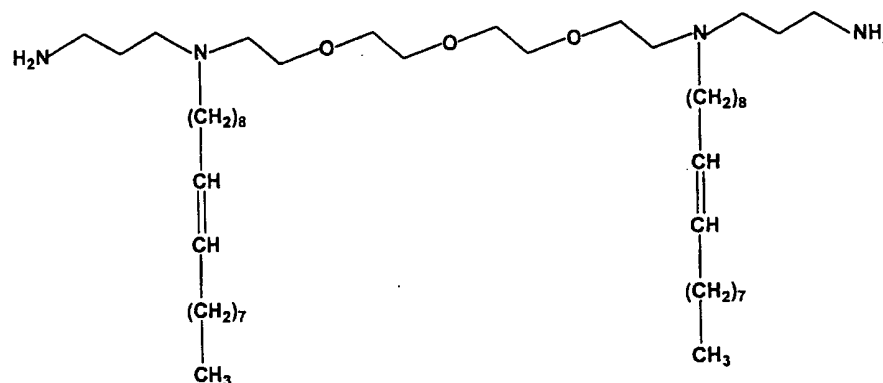
i and j are integers from about 2 to about 3; and

k is an integer from 1 to about 3.

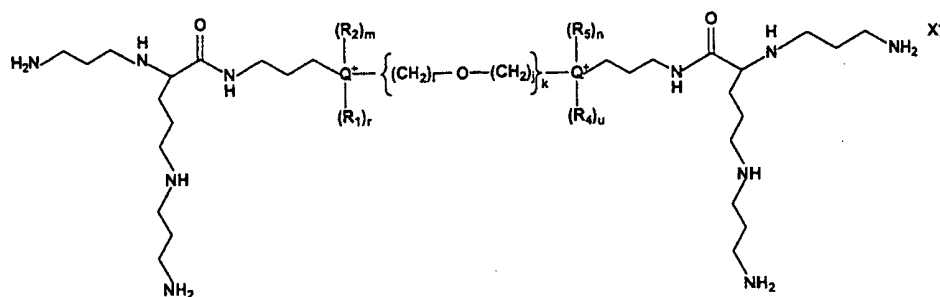
- 15 43. The compound as claimed in claim 42, which is:



44. The compound as claimed in claim 42, which is:



5 45. The compound as claimed in claim 1, wherein said compound has the formula:



10 wherein

Q, R₁, R₄, r, u, m and n are as defined in claim 1;

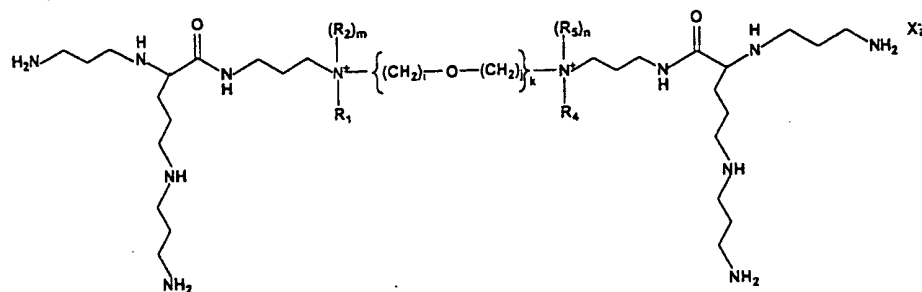
R₂ and R₅, independently of one another, are selected from the group consisting of H and a C₁-C₈ alkyl, alkenyl, aryl, and alkyl optionally substituted by one or more of an alcohol, an amine, an amide, an ether, a polyether, a polyamide, an ester, a mercaptan,

15 a urea, a thiourea, a guanidyl, or a carbamoyl group;

i and j are integers from about 2 to about 3; and

k is an integer from 1 to about 3.

46. The compound as claimed in claim 5, wherein said compound has the formula:



5

wherein

at least one of R_1 and R_4 is a straight chain or branched, cyclic, alkyl, alkenyl, alkynyl or aryl group having from about 8 to about 24 carbon atoms;

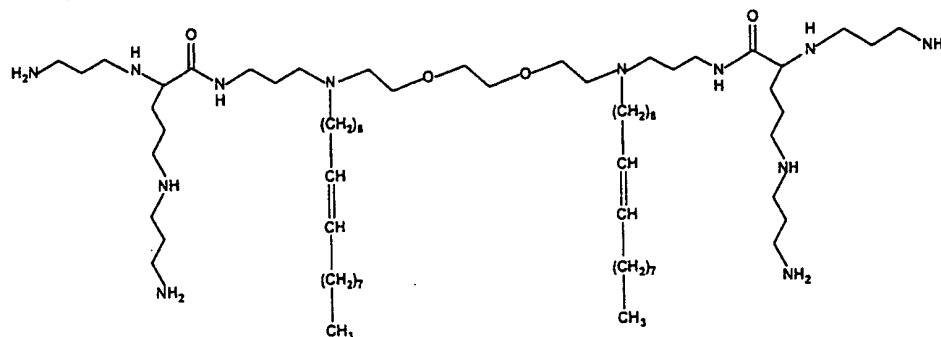
R_2 and R_5 , independently of one another, are selected from the group consisting of H and a C_1 - C_8 alkyl, alkenyl, aryl, and alkyl optionally substituted by one or more of an alcohol, an amine, an amide, an ether, a polyether, a polyamide, an ester, a mercaptan, a urea, a thiourea, a guanidyl, or a carbamoyl group;

m and n are 0 or 1;

i and j are integers from about 2 to about 3; and

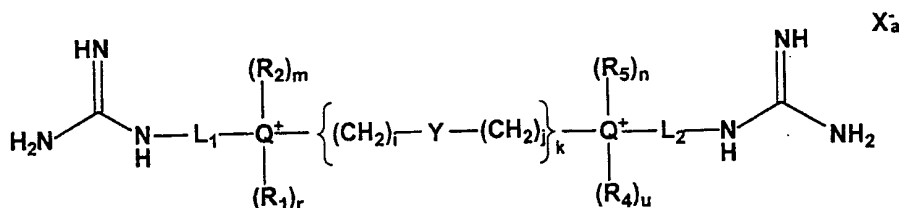
15 k is an integer from 1 to about 3.

47. The compound as claimed in claim 46, which is:



5

48. The compound as claimed in claim 1, wherein said compound has the formula:



10

wherein

Q, R₁, R₄, r, u, m and n are as defined in claim 1;

R₂ and R₅, independently of one another, are selected from the group consisting of H and a C₁ - C₈ alkyl, alkenyl, aryl, and alkyl optionally substituted by one or more of an alcohol, an amine, an amide, an ether, a polyether, a polyamide, an ester, a mercaptan, a urea, a thiourea, a guanidyl, or a carbamoyl group;

i and j are integers from about 2 to about 3;

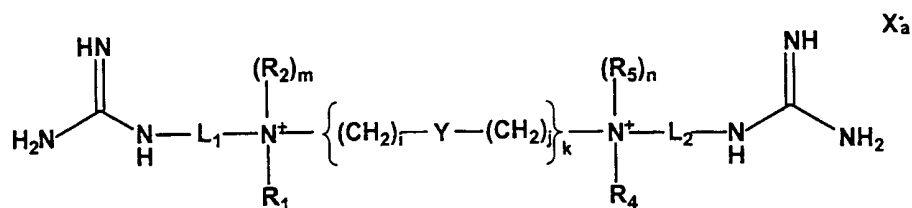
k is an integer from 1 to about 3;

L₁ and L₂, independently from one another, are an alkylene or an alkylene ether;

20 and

Y is selected from the group consisting of CH₂, O, S and NH.

49. The compound as claimed in claim 5, wherein said compound has the formula:



wherein

- 5 at least one of R_1 and R_4 is a straight chain or branched, cyclic, alkyl, alkenyl, alkynyl or aryl group having from about 8 to about 24 carbon atoms;

- R_2 and R_5 , independently of one another, are selected from the group consisting of H and a $\text{C}_1 - \text{C}_8$ alkyl, alkenyl, aryl, and alkyl optionally substituted by one or more of an alcohol, an amine, an amide, an ether, a polyether, a polyamide, an ester, a mercaptan,
 10 a urea, a thiourea, a guanidyl, or a carbamoyl group;

m and n are 0 or 1;

i and j are integers from about 2 to about 3;

k is an integer from 1 to about 3;

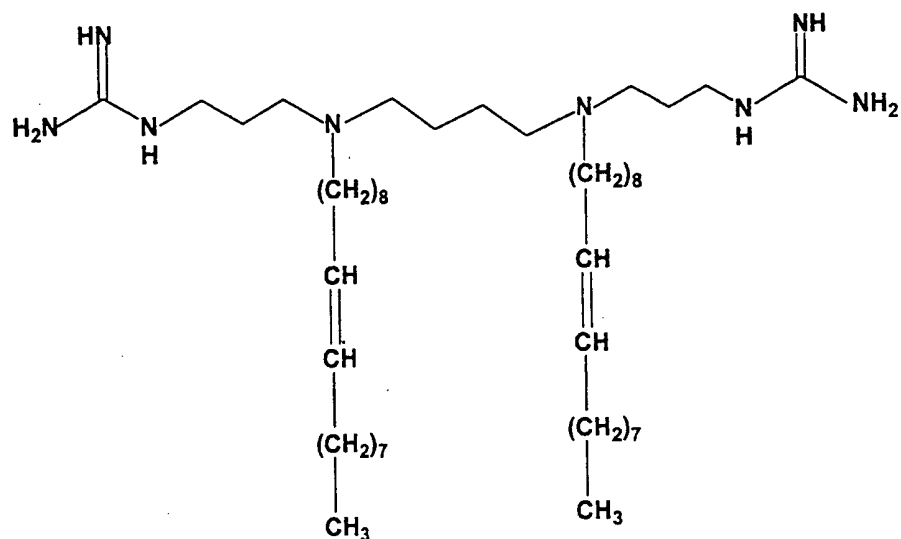
L_1 and L_2 , independently from one another, are an alkylene or an alkylene ether;

- 15 and

Y is selected from the group consisting of CH_2 , O, S and NH.

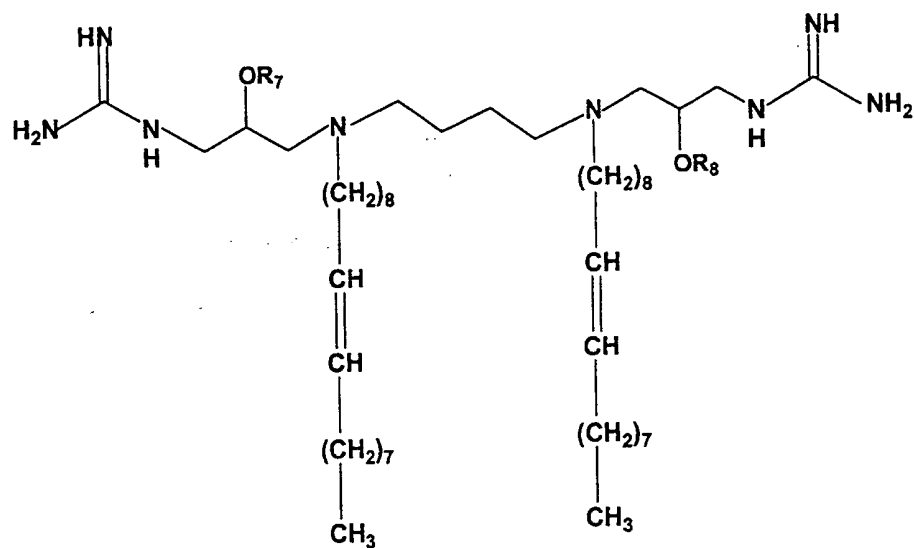
50. The compound as claimed in claim 49, which is:

-98-



51. The compound as claimed in claim 49, which is:

5



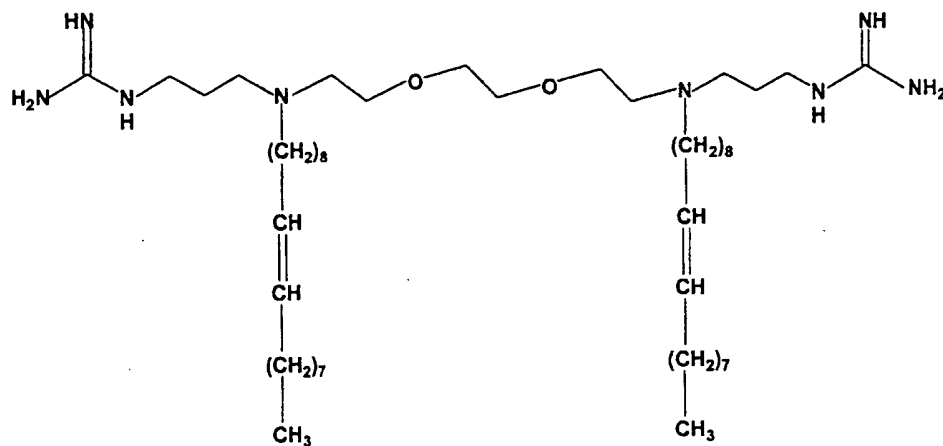
wherein R_7 and R_8 are independently H or a carbohydrate.

10

52. The compound as claimed in claim 51, wherein R_7 and R_8 are H.

-99-

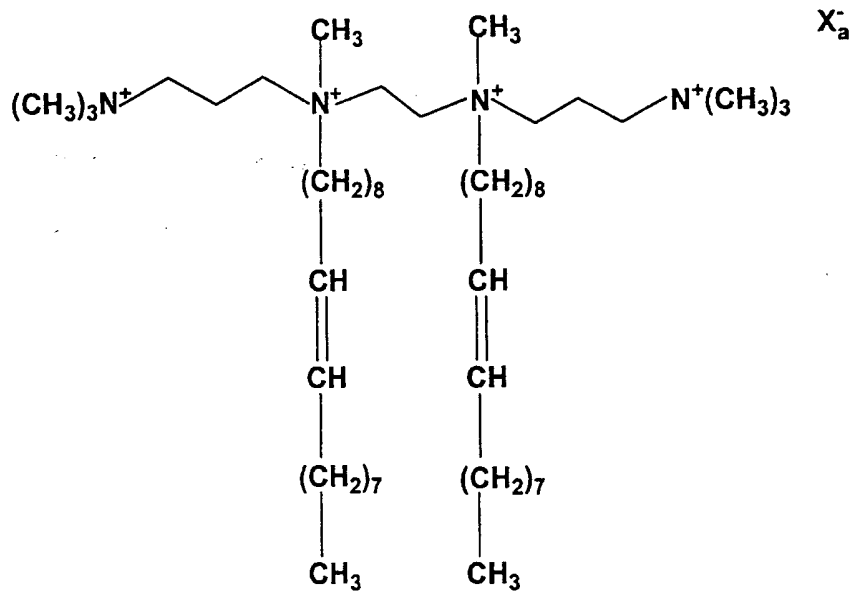
53. The compound as claimed in claim 49, which is:



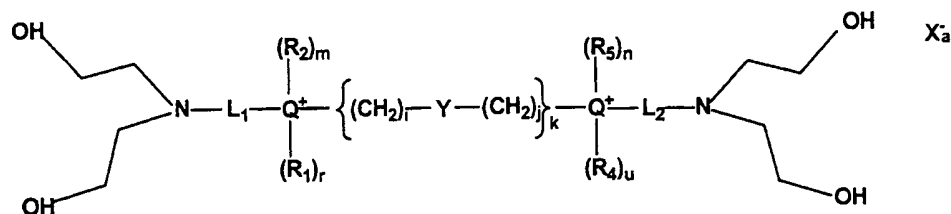
5

54. The compound as claimed in claim 5, which is:

10



55. The compound as claimed in claim 1, wherein said compound has the formula:



5 wherein

Q, R₁, R₄, r, u, m and n are as defined in claim 1;

R₂ and R₅, independently of one another, are selected from the group consisting of H and a C₁ - C₈ alkyl, alkenyl, aryl, and alkyl optionally substituted by one or more of an alcohol, an amine, an amide, an ether, a polyether, a polyamide, an ester, a mercaptan, a urea, a thiourea, a guanidyl, or a carbamoyl group;

i and j are integers from about 2 to about 3;

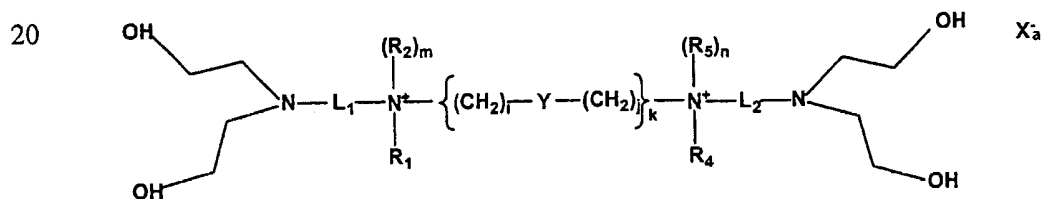
k is an integer from 1 to about 3;

L₁ and L₂, independently from one another, are an alkylene or an alkylene ether;

and

15 Y is selected from the group consisting of CH₂, O, S and NH.

56. The compound as claimed in claim 5, wherein said compound has the formula:



wherein

at least one of R₁ and R₄ is a straight chain or branched, cyclic, alkyl, alkenyl, alkynyl or aryl group having from about 8 to about 24 carbon atoms;

R₂ and R₅, independently of one another, are selected from the group consisting of H and a C₁ - C₈ alkyl, alkenyl, aryl, and alkyl optionally substituted by one or more of an alcohol, an amine, an amide, an ether, a polyether, a polyamide, an ester, a mercaptan, a urea, a thiourea, a guanidyl, or a carbamoyl group;

-101-

m and n are 0 or 1;

i and j are integers from about 2 to about 3;

k is an integer from 1 to about 3;

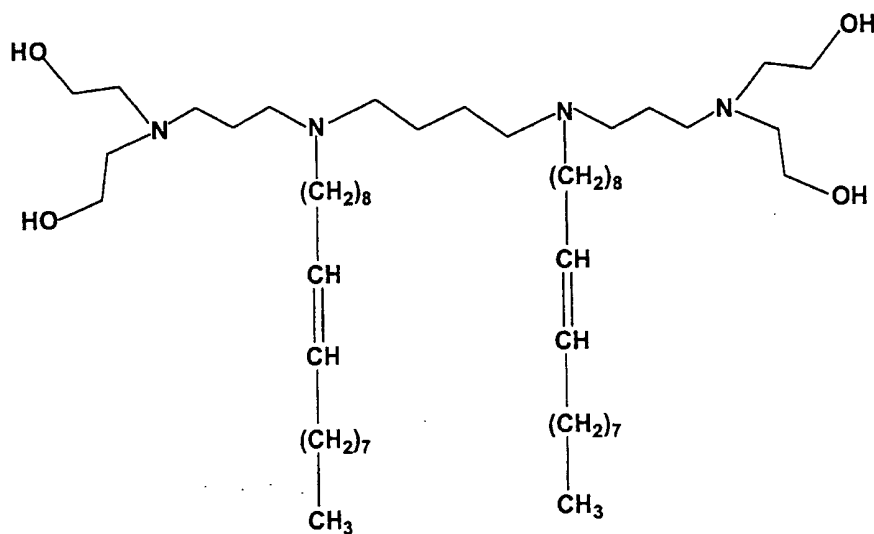
L_1 and L_2 , independently from one another, are an alkylene or an alkylene ether;

5 and

Y is selected from the group consisting of CH_2 , O, S and NH.

57. The compound as claimed in claim 56, which is:

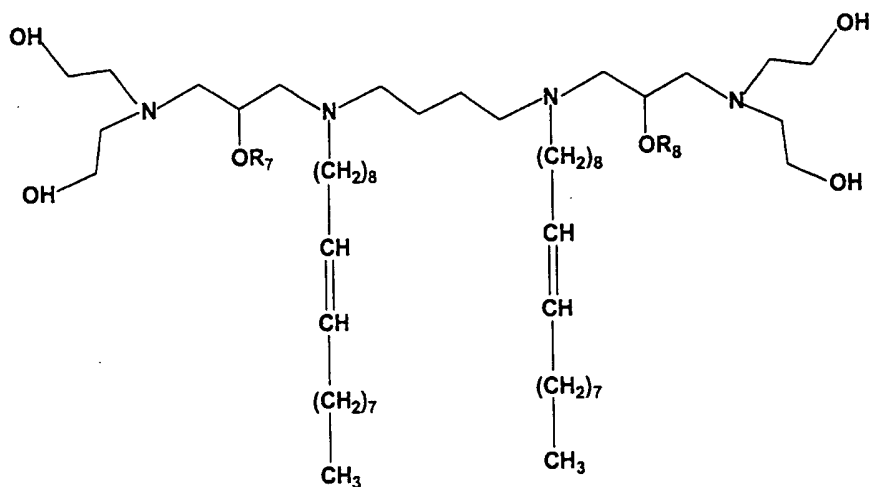
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58. The compound as claimed in 56, which is:

15

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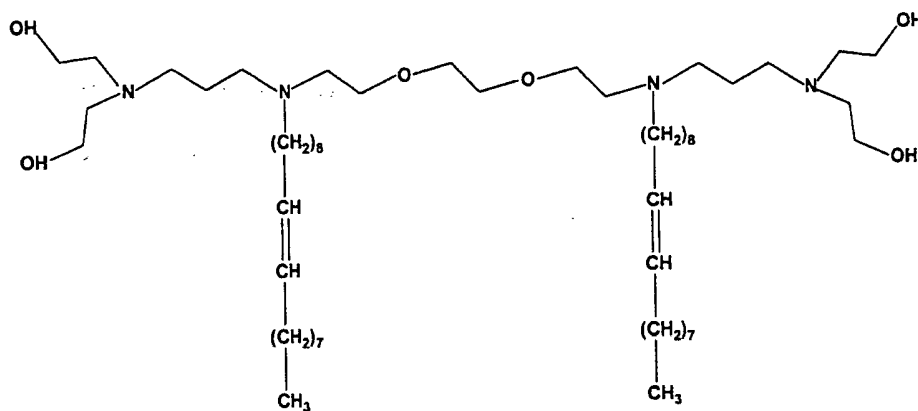
wherein R_7 and R_8 are independently H or a carbohydrate.

5

59. The compound as claimed in claim 58, wherein R_7 and R_8 are H.

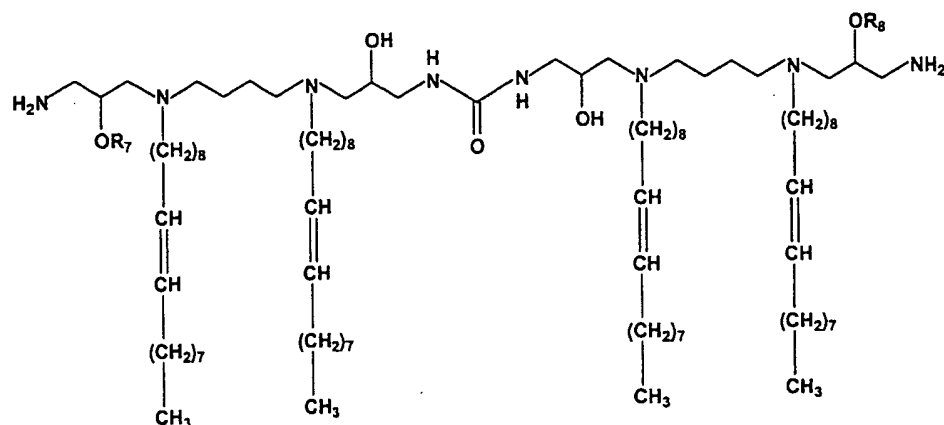
60. The compound as claimed in 56, which is:

10



61. The compound as claimed in claim 5, which is:

-103-



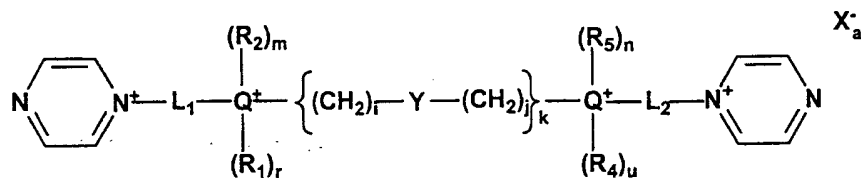
wherein R_7 and R_8 are independently H or a carbohydrate.

5

62. The compound as claimed in claim 61, wherein R_7 and R_8 are H.

63. The compound as claimed in claim 1, wherein said compound has the formula:

10



wherein

Q , R_1 , R_4 , r , u , m and n are as defined in claim 1;

15

R_2 and R_5 , independently of one another, are selected from the group consisting of H and a $C_1 - C_8$ alkyl, alkenyl, aryl, and alkyl optionally substituted by one or more of an alcohol, an amine, an amide, an ether, a polyether, a polyamide, an ester, a mercaptan, a urea, a thiourea, a guanidyl, or a carbamoyl group;

i and j are integers from about 2 to about 3;

20

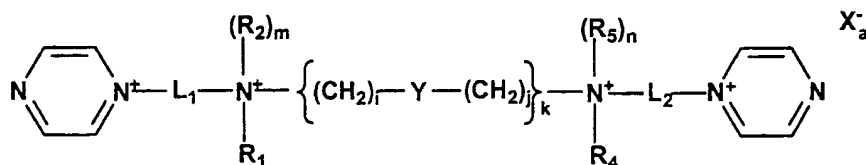
k is an integer from 1 to about 3;

L_1 and L_2 , independently from one another, are an alkylene or an alkylene ether;

and

Y is selected from the group consisting of CH_2 , O, S and NH.

64. The compound as claimed in claim 5, wherein said compound has the formula:



5

wherein

at least one of R_1 and R_4 is a straight chain or branched, cyclic, alkyl, alkenyl, alkynyl or aryl group having from about 8 to about 24 carbon atoms;

R_2 and R_5 , independently of one another, are selected from the group consisting of H and a $\text{C}_1 - \text{C}_8$ alkyl, alkenyl, aryl, and alkyl optionally substituted by one or more of an alcohol, an amine, an amide, an ether, a polyether, a polyamide, an ester, a mercaptan, a urea, a thiourea, a guanidyl, or a carbamoyl group;

m and n are 0 or 1;

i and j are integers from about 2 to about 3;

15 k is an integer from 1 to about 3;

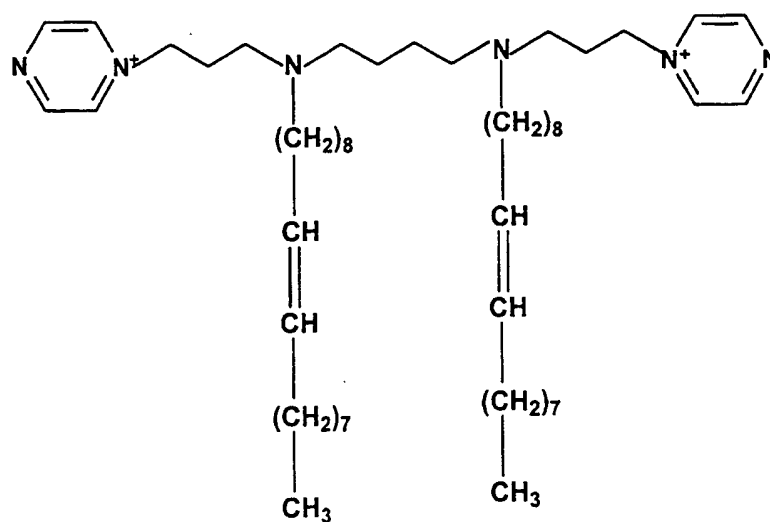
L_1 and L_2 , independently from one another, are an alkylene or an alkylene ether;

and

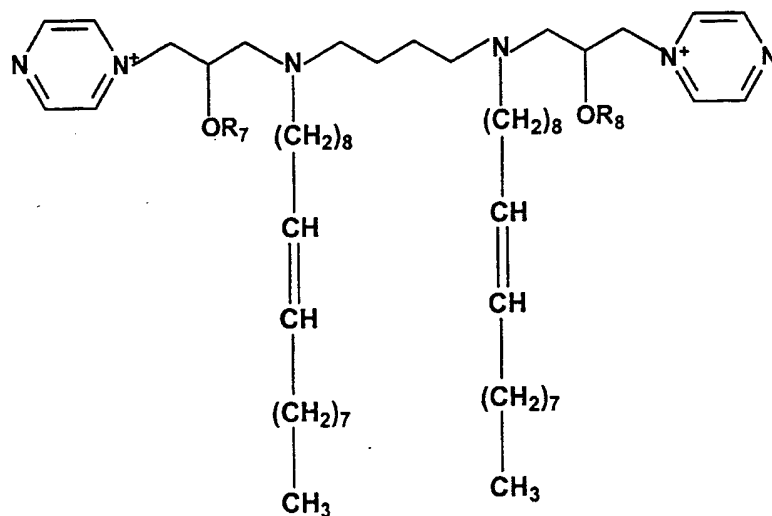
Y is selected from the group consisting of CH_2 , O, S and NH.

20 65. The compound as claimed in claim 64, which is:

-105-



- 5 66. The compound as claimed in claim 64, which is:

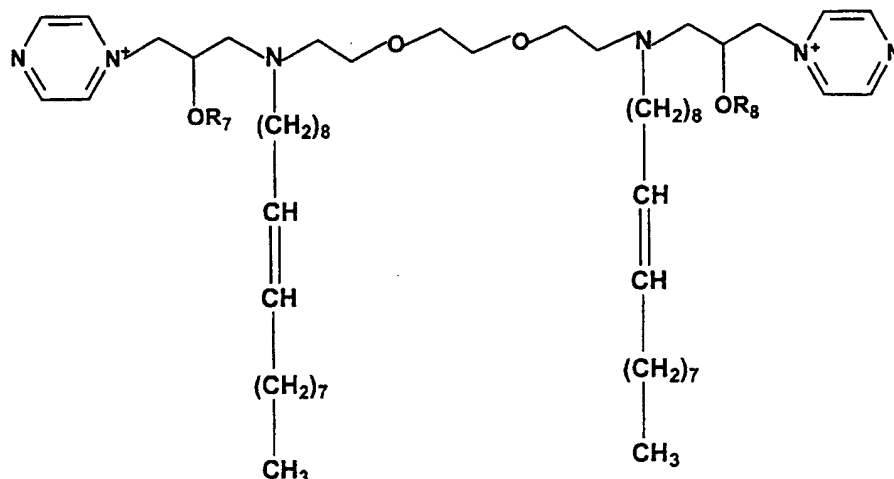


10

wherein R_7 and R_8 are independently H or a carbohydrate.

67. The compound as claimed in claim 66, wherein R_7 and R_8 are H.

- 15 68. The compound as claimed in claim 64, which is:

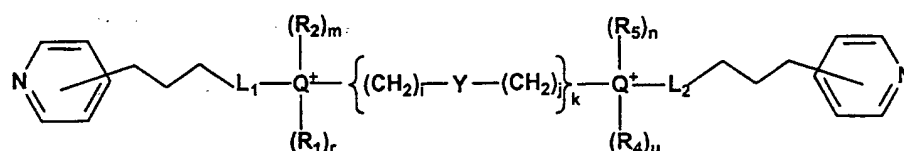


5 wherein R_7 and R_8 are independently H or a carbohydrate.

69. The compound as claimed in claim 68, wherein R_7 and R_8 are H.

70. The compound as claimed in claim 1, wherein said compound has the formula:

10



wherein

15 Q , R_1 , R_2 , r , u , m and n are as defined in claim 1;

R_2 and R_5 , independently of one another, are selected from the group consisting of H and a $C_1 - C_8$ alkyl, alkenyl, aryl, and alkyl optionally substituted by one or more of an alcohol, an amine, an amide, an ether, a polyether, a polyamide, an ester, a mercaptan, a urea, a thiourea, a guanidyl, or a carbamoyl group;

20 i and j are integers from about 2 to about 3;

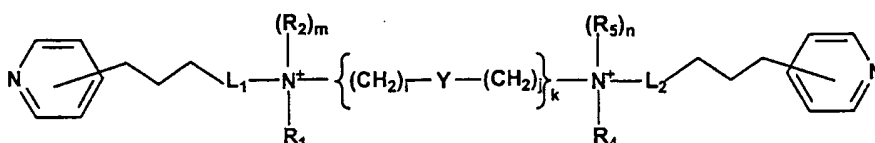
k is an integer from 1 to about 3;

-107-

L_1 and L_2 , independently from one another, are an alkylene or an alkylene ether;
and

Y is selected from the group consisting of CH_2 , O, S and NH.

5 71. The compound as claimed in claim 5, wherein said compound has the formula:



10

wherein

at least one of R_1 and R_4 is a straight chain or branched, cyclic, alkyl, alkenyl, alkynyl or aryl group having from about 8 to about 24 carbon atoms;

15 R_2 and R_5 , independently of one another, are selected from the group consisting of H and a $C_1 - C_8$ alkyl, alkenyl, aryl, and alkyl optionally substituted by one or more of an alcohol, an amine, an amide, an ether, a polyether, a polyamide, an ester, a mercaptan, a urea, a thiourea, a guanidyl, or a carbamoyl group;

m and n are 0 or 1;

i and j are integers from about 2 to about 3;

20 k is an integer from 1 to about 3;

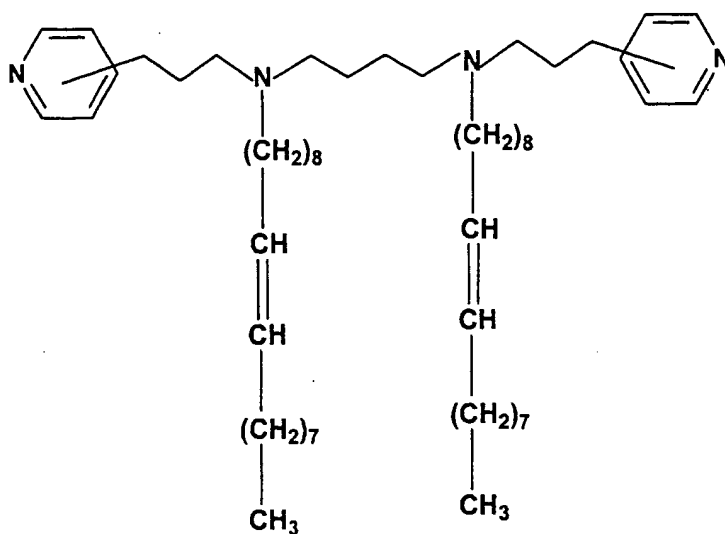
L_1 and L_2 , independently from one another, are an alkylene or an alkylene ether;

and

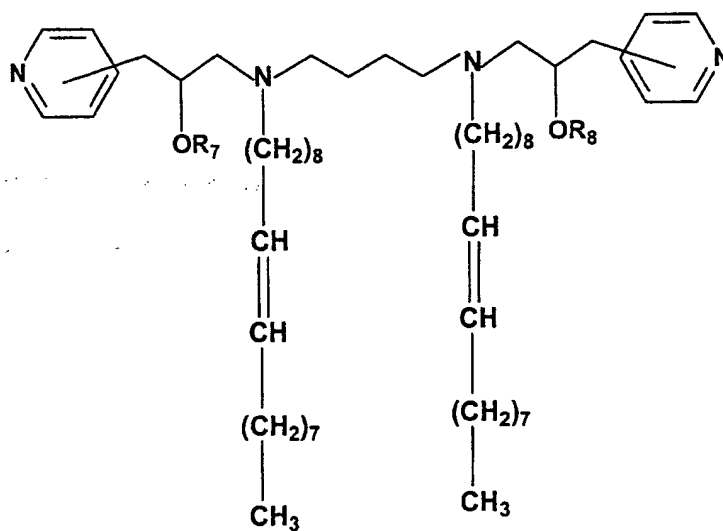
Y is selected from the group consisting of CH_2 , O, S and NH.

25 72. The compound as claimed in claim 71, which is:

-108-



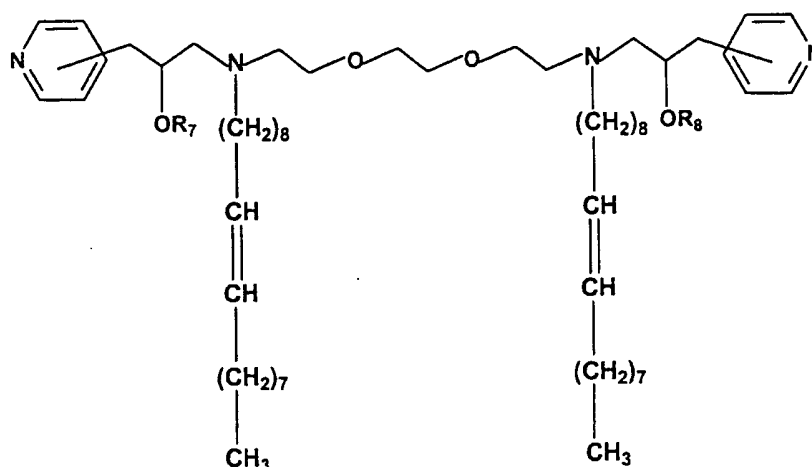
- 5 73. The compound as claimed in claim 71, which is:



- 10 wherein R_7 and R_8 are independently H or a carbohydrate.

74. The compound according to claim 73, wherein R_7 and R_8 are H.

75. The compound as claimed in claim 71, which is:

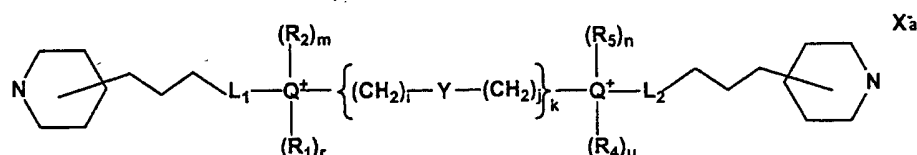


5

wherein R_7 and R_8 independently are H or a carbohydrate.

76. The compound as claimed in claim 75, wherein R_7 and R_8 are H.

10 77. The compound as claimed in claim 1, wherein said compound has the formula:



15 wherein

Q , R_1 , R_4 , r , u , m , and n are as defined in claim 1;

R_2 and R_3 , independently of one another, are selected from the group consisting of H and a $C_1 - C_8$ alkyl, alkenyl, aryl, and alkyl optionally substituted by one or more of an alcohol, an amine, an amide, an ether, a polyether, a polyamide, an ester, a mercaptan,

20 a urea, a thiourea, a guanidyl, or a carbamoyl group;

i and j are integers from about 2 to about 3;

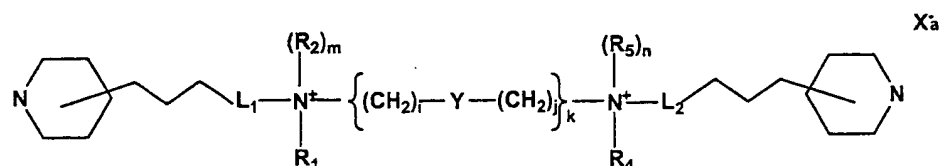
k is an integer from 1 to about 3;

-110-

L_1 and L_2 , independently from one another, are an alkylene or an alkylene ether;
and

Y is selected from the group consisting of CH_2 , O, S and NH.

- 5 78. The compound as claimed in claim 5, wherein said compound has the formula:



10 wherein

at least one of R_1 and R_4 is a straight chain or branched, cyclic, alkyl, alkenyl, alkynyl or aryl group having from about 8 to about 24 carbon atoms;

R_2 and R_5 , independently of one another, are selected from the group consisting of H and a C_1 - C_8 alkyl, alkenyl, aryl, and alkyl optionally substituted by one or more of
15 an alcohol, an amine, an amide, an ether, a polyether, a polyamide, an ester, a mercaptan, a urea, a thiourea, a guanidyl, or a carbamoyl group;

m and n are 0 or 1;

i and j are integers from about 2 to about 3;

k is an integer from 1 to about 3;

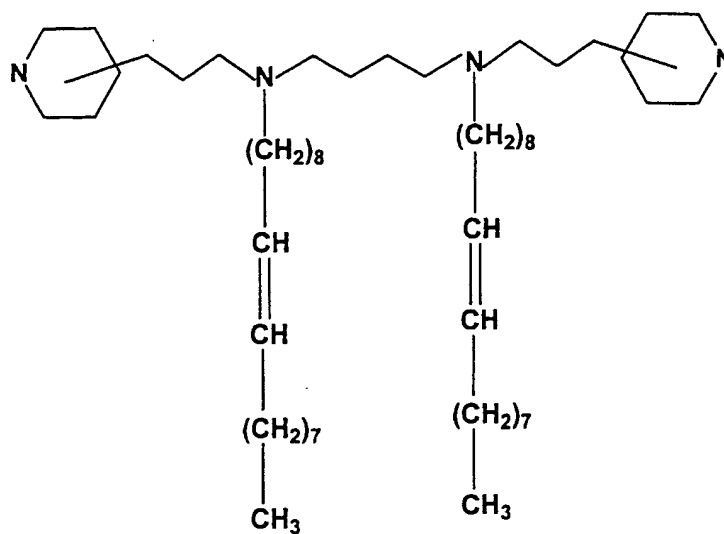
20 L_1 and L_2 , independently from one another, are an alkylene or an alkylene ether;
and

Y is selected from the group consisting of CH_2 , O, S and NH.

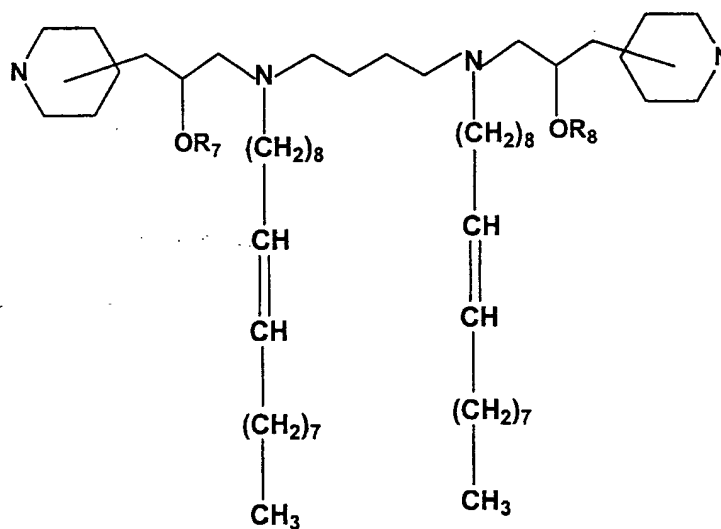
79. The compound as claimed in claim 78, which is:

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-111-



- 5 80. The compound as claimed in claim 78, which is:

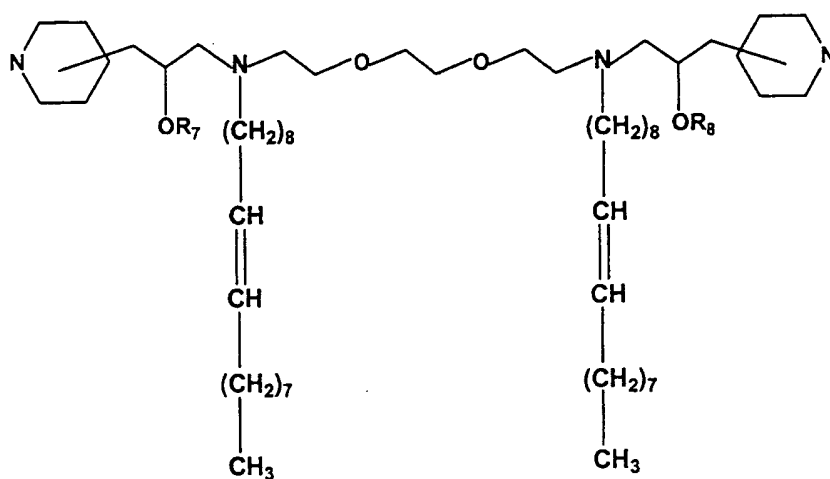


wherein R_7 and R_8 are independently H or a carbohydrate.

- 10 81. The compound as claimed in claim 80, wherein R_7 and R_8 are H.

82. The compound as claimed in claim 78, which is:

-112-



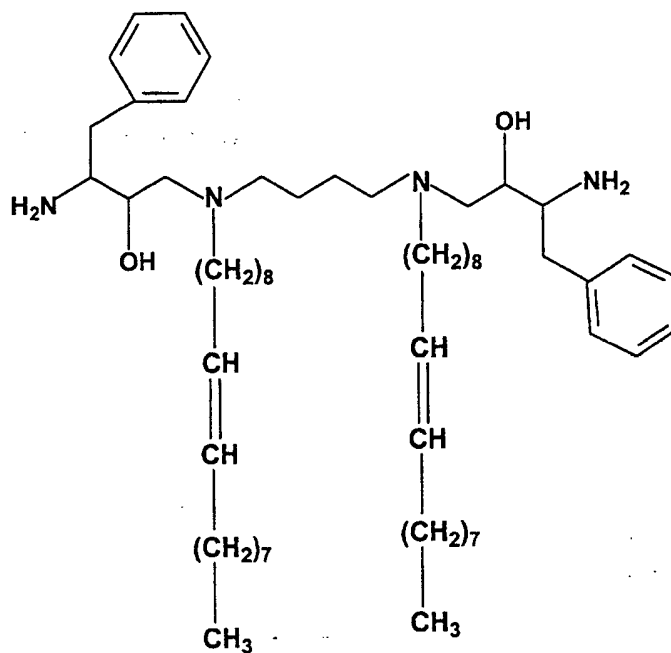
wherein R_7 and R_8 are independently H or a carbohydrate.

5

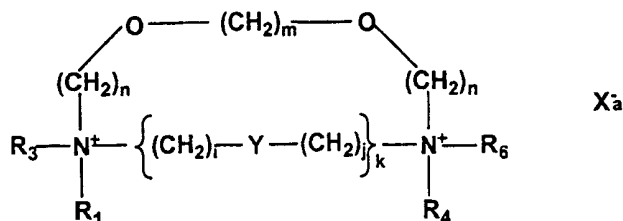
83. The compound as claimed in claim 82, wherein R_7 and R_8 are H.

84. The compound as claimed in claim 5, which is:

10



85. The compound as claimed in claim 1, wherein said compound has the formula:



5 wherein

Y is selected from the group consisting of CH₂, an ether, a polyether, an amide, a polyamide, an ester, a sulfide, a urea, a thiourea, a guanidyl, a carbamoyl, a carbonate, a phosphate, a sulfate, a sulfoxide, an imine, a carbonyl, and a secondary amino group and wherein Y is optionally substituted by -X₁-L'-X₂-Z or -Z;

10 R₁, R₃, R₄ and R₆, independently of one another, are selected from the group consisting of H, -(CH₂)_p-D-Z, an alkyl, an alkenyl, an aryl, and an alkyl or an alkyl ether optionally substituted by one or more of an alcohol, an aminoalcohol, an amine, an amide, an ether, a polyether, a polyamide, an ester, a mercaptan, an alkylthio, a urea, a thiourea, a guanidyl, or a carbamoyl group, and at least one of R₁, R₃, R₄ and R₆ is a
15 straight chain or branched, cyclic, alkyl, alkenyl, alkynyl or aryl group having from 6 to about 64 carbon atoms; and R₁, R₃, R₄ and R₆ may optionally be covalently linked with each other or with Y, to form a cyclic moiety;

Z is selected from the group consisting of amine, spermiyl, carboxyspermiyl, guanidyl, spermidinyl, putricinyl, diaminoalkyl, pyridyl, piperidinyl, pyrrolidinyl,
20 polyamine, amino acid, peptide, and protein;

X₁ and X₂, independently of one another, are selected from the group consisting of NH, O, S, alkylene, and arylene;

L' is selected from the group consisting of alkylene, alkenylene, alkynylene, arylene, alkylene ether, and polyether;

25 D is Q or a bond;

m and n are 0 or 1; and

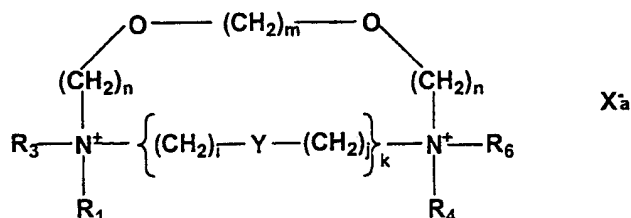
i, j, k, l and p are integers from 1 to about 10.

86. The compound as claimed in claim 85, wherein at least one of R_1 and R_4 is a straight chain or branched, cyclic, alkyl, alkenyl, alkynyl or aryl group having from about 8 to about 24 carbon atoms.

5 87. The compound as claimed in claim 85, wherein the alkyl ether optionally substituted by one or more alcohol groups is a carbohydrate.

88. The compound as claimed in claim 87, wherein the carbohydrate is selected from the group consisting of galactose, fructose, glucose, maltose, sucrose, cellobiose, lactose,
10 mannose, glucopyranose, mannopyranose and galactopyranose.

89. The compound as claimed in claim 85, wherein said compound has the formula:



15 wherein

Y is selected from the group consisting of CH_2 , an ether, a polyether, an amide, a polyamide, an ester, a sulfide, a urea, a thiourea, a guanidyl, a carbamoyl, a carbonate, and a secondary amino group;

R_1 , R_3 , R_4 and R_6 , independently of one another, are selected from the group
20 consisting of H, $-(\text{CH}_2)_p$ -Z, an alkyl, an alkenyl, an aryl, and an alkyl or an alkyl ether optionally substituted by one or more of an alcohol, an aminoalcohol, an amine, an amide, an ether, a polyether, a polyamide, an ester, a mercaptan, a urea, a thiourea, a guanidyl, or a carbamoyl group, and at least one of R_1 , R_3 , R_4 and R_6 is a straight chain
25 carbon atoms; and R_1 , R_3 , R_4 and R_6 may optionally be covalently linked with each other, to form a cyclic moiety;

Z is selected from the group consisting of amine, spermiyl, carboxyspermiyl, guanidyl, spermidinyl, putricinyl, diaminoalkyl, pyridyl, piperidinyl, pyrrolidinyl, polyamine, amino acid, peptide, and protein;

-115-

m and n are 0 or 1; and

i, j, k, l and p are integers from 1 to about 10.

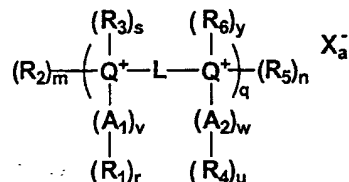
90. The compound as claimed in claim 89, wherein at least one of R_1 and R_4 is a straight chain or branched, cyclic, alkyl, alkenyl, alkynyl or aryl group having from about 8 to about 24 carbon atoms.

91. The compound as claimed in claim 89, wherein the alkyl ether optionally substituted by one or more alcohol groups is a carbohydrate.

10

92. The compound as claimed in claim 91, wherein the carbohydrate is selected from the group consisting of galactose, fructose, glucose, maltose, sucrose, cellobiose, lactose, mannose, glucopyranose, mannopyranose and galactopyranose.

15 93. A compound having the formula:



wherein

Q is selected from the group consisting of N, O and S;

20 L is a bivalent organic radical capable of covalently linking each Q;

$R_1 - R_6$, independently of one another, are selected from the group consisting of H, $-(CH_2)_p-D-Z$, an alkyl, an alkenyl, an aryl, and an alkyl or alkyl ether optionally substituted by one or more of an alcohol, an aminoalcohol, an amine, an amide, an ether, a polyether, a polyamide, an ester, a mercaptan, an alkylthio, a urea, a thiourea, a guanidyl, or a carbamoyl group, and wherein at least one of R_1 , R_3 , R_4 and R_6 is a straight chain or branched, cyclic, alkyl, alkenyl, alkynyl or aryl group having from about 6 to about 64 carbon atoms; and R_1 and R_4 or R_3 and R_6 may optionally be covalently linked with each other, or with L to form a cyclic moiety;

-116-

Z is selected from the group consisting of amine, spermiyl, carboxyspermiyl, guanidyl, spermidinyl, putricinyl, diaminoalkyl, pyridyl, piperidinyl, pyrrolidinyl, polyamine, amino acid, peptide, and protein;

D is Q or a bond;

5 A₁ and A₂, independently of one another, are selected from the group consisting of CH₂O, CH₂S, CH₂NH, C(O), C(NH), C(S) and (CH₂)_i;

X is a physiologically acceptable anion;

m, n, r, s, u, v, w and y are 0 or 1, with the proviso that when both m and n are 0 at least one of r, s, u and y is other than 0;

10 i, j, k, l, p and t are integers from 0 to about 100;

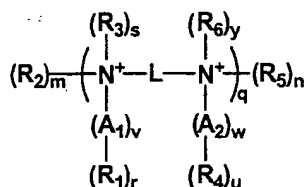
q is an integer from 1 to about 1000; and

a is the number of positive charge divided by the valence of the anion.

94. The compound as claimed in any one of claims 1, 2, 5, 6, 9, 11, 12, 15, 16, 20,
15 21, 22, 29, 32, 41, 42, 45, 46, 48, 49, 55, 56, 63, 64, 70, 71, 77, 78, 85, 86, 87, 88, 89, 90, 91, 92 and 93, wherein said cyclic group is a cholesteryl group.

95. A compound or a polycation having the formula:

20



wherein

L is C, CH, (CH₂)_i, or {(CH₂)_i - Y - (CH₂)_j}_k, wherein Y is selected from the group consisting of CH₂, an ether, a polyether, an amide, a polyamide, an ester, a sulfide, a urea, a thiourea, a guanidyl, a carbamoyl, a carbonate, a phosphate, a sulfate, a sulfoxide, an
25 imine, a carbonyl, and a secondary amino group and wherein Y is optionally substituted by -X₁-L'-X₂-Z or -Z;

R₁ - R₆, independently of one another, are selected from the group consisting of H, -(CH₂)_p-D-Z, an alkyl, an alkenyl, an aryl, and an alkyl or an alkyl ether optionally substituted by one or more of an alcohol, an aminoalcohol, an amine, an amide, an ether,

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a polyether, a polyamide, an ester, a mercaptan, an alkylthio, a urea, a thiourea, a guanidyl, or a carbamoyl group, and wherein at least one of R_1 , R_3 , R_4 and R_6 is a straight chain or branched, cyclic, alkyl, alkenyl, alkynyl or aryl group having from about 6 to about 64 carbon atoms;

- 5 Z is selected from the group consisting of amine, spermyl, carboxyspermyl, guanidyl, spermidinyl, putricinyl, diaminoalkyl, pyridyl, piperidinyl, pyrrolidinyl, polyamine, amino acid, amino acid derivative, peptide, and protein;

X_1 and X_2 , independently of one another, are selected from the group consisting of NH, O, S, alkylene and arylene;

- 10 L' is selected from the group consisting of alkylene, alkenylene, alkynylene, arylene, alkylene ether, and polyether;

A_1 and A_2 , independently of one another, are selected from the group consisting of CH_2O , CH_2S , CH_2NH , $C(O)$, $C(NH)$, $C(S)$ and $(CH_2)_i$;

- 15 m, n, r, s, u, v, w and y are 0 or 1, with the proviso that when both m and n are 0 at least one of r, s, u and y is other than 0;

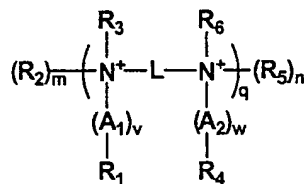
i, j, k, l, p and t are integers from 0 to about 100; and

q is an integer from 1 to about 1000.

96. The compound or the polycation as claimed in claim 95, wherein at least one of
20 R_1 , R_3 , R_4 and R_6 is a straight chain or branched, cyclic, alkyl, alkenyl, alkynyl or aryl group having from about 8 to about 24 carbon atoms.

97. A compound or a polycation having the formula:

25



wherein

L is $(\text{CH}_2)_1$ or $\{(\text{CH}_2)_i - \text{Y} - (\text{CH}_2)_j\}_k$, wherein Y is selected from the group consisting of CH_2 , an ether, a polyether, an amide, a polyamide, an ester, a sulfide, a urea, a thiourea, a guanidyl, a carbamoyl, a carbonate, and a secondary amino group;

5 $\text{R}_1 - \text{R}_6$, independently of one another, are selected from the group consisting of H, $-(\text{CH}_2)_p-\text{Z}$, an alkyl, an alkenyl, an aryl, and an alkyl or an alkyl ether optionally substituted by one or more of an alcohol, an aminoalcohol, an amine, an amide, an ether, a polyether, a polyamide, an ester, a mercaptan, a urea, a thiourea, a guanidyl, or a carbamoyl group, and at least one of R_1 , R_3 , R_4 and R_6 is a straight chain or branched,
10 cyclic, alkyl, alkenyl, alkynyl or aryl group having from about 6 to about 64 carbon atoms;

Z is selected from the group consisting of amine, spermyl, carboxyspermyl, guanidyl, spermidinyl, putricinyl, diaminoalkyl, pyridyl, piperidinyl, pyrrolidinyl, polyamine, amino acid, amino acid derivative, peptide, and protein;

15 A_1 and A_2 , independently of one another, are selected from the group consisting of CH_2O , CH_2S , CH_2NH , $\text{C}(\text{O})$, $\text{C}(\text{NH})$, $\text{C}(\text{S})$ and $(\text{CH}_2)_i$;

m, n, v and w are 0 or 1;

i, j, k, l, p and t are integers from 1 to about 100; and

q is an integer from 1 to about 1000.

20

98. The compound or the polycation as claimed in claim 97, wherein at least one of R_1 , R_3 , R_4 and R_6 is a straight chain or branched, cyclic, alkyl, alkenyl, alkynyl or aryl group having from about 8 to about 24 carbon atoms.

25 99. The compound or the polycation as claimed in claim 95 or 97, wherein the alkyl ether optionally substituted by one or more alcohol groups is a carbohydrate.

100. The compound or the polycation as claimed in claim 99, wherein the carbohydrate is selected from the group consisting of galactose, fructose, glucose, maltose, sucrose,
30 cellobiose, lactose, mannose, glucopyranose, mannopyranose and galactopyranose.

101. A composition comprising one or more compounds of any one of claims 1 to 93.
102. A composition comprising one or more compounds of any one of claims 1 to 93 and at least one additional component selected from the group consisting of a cell, cells,
5 a cell culture, a cell culture media, a neutral lipid, a nucleic acid, and a transfection enhancer.
103. The composition of claim 102, which comprises a nucleic acid.
- 10 104. A lipid aggregate comprising one or more compounds of any one of claims 1 to 93.
105. The lipid aggregate of claim 104, which comprises at least one lipid aggregate-forming compound.
- 15 106. The lipid aggregate of claim 105, wherein said lipid aggregate-forming compound is selected from the group consisting of DOPE, DOPC and cholesterol.
107. A kit comprising one or more compounds of any one of claims 1 to 93 and at least
20 one additional component selected from the group consisting of a cell, cells, a cell culture media, a nucleic acid, a transfection enhancer and instructions for transfecting a cell or cells.
108. A method for introducing a polyanion into a cell or cells, said method comprising
25 forming a liposome from a positively charged compound of any one of claims 1 to 93, contacting the liposome with a polyanion to form a positively-charged polyanion-liposome complex and incubating the complex with a cell or cells.
109. A method for introducing a biologically active substance into a cell, said method
30 comprising forming a liposome of a compound of any one of claims 1 to 93 and a biologically active substance and incubating the liposome with a cell or cell culture.

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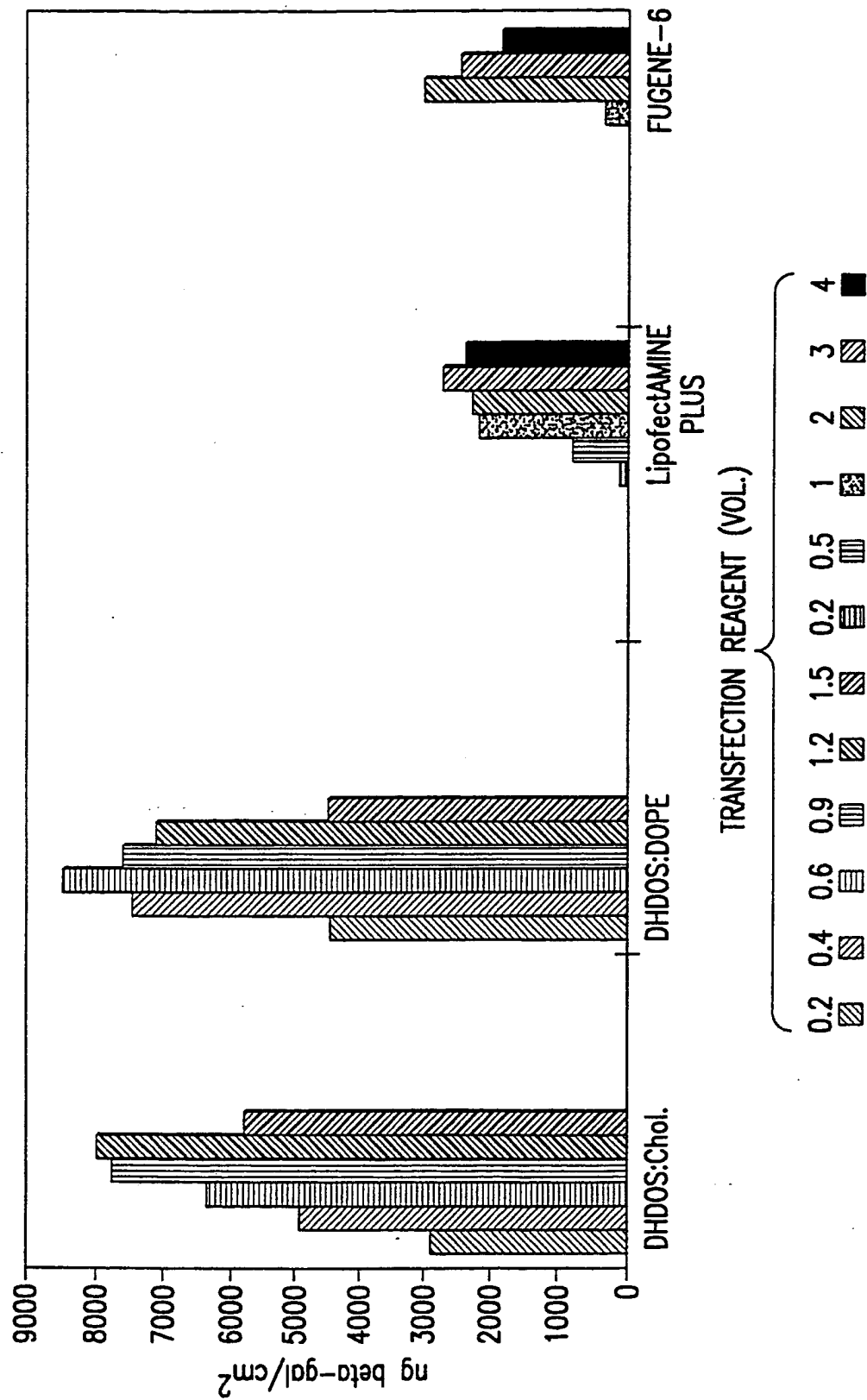


FIG.1

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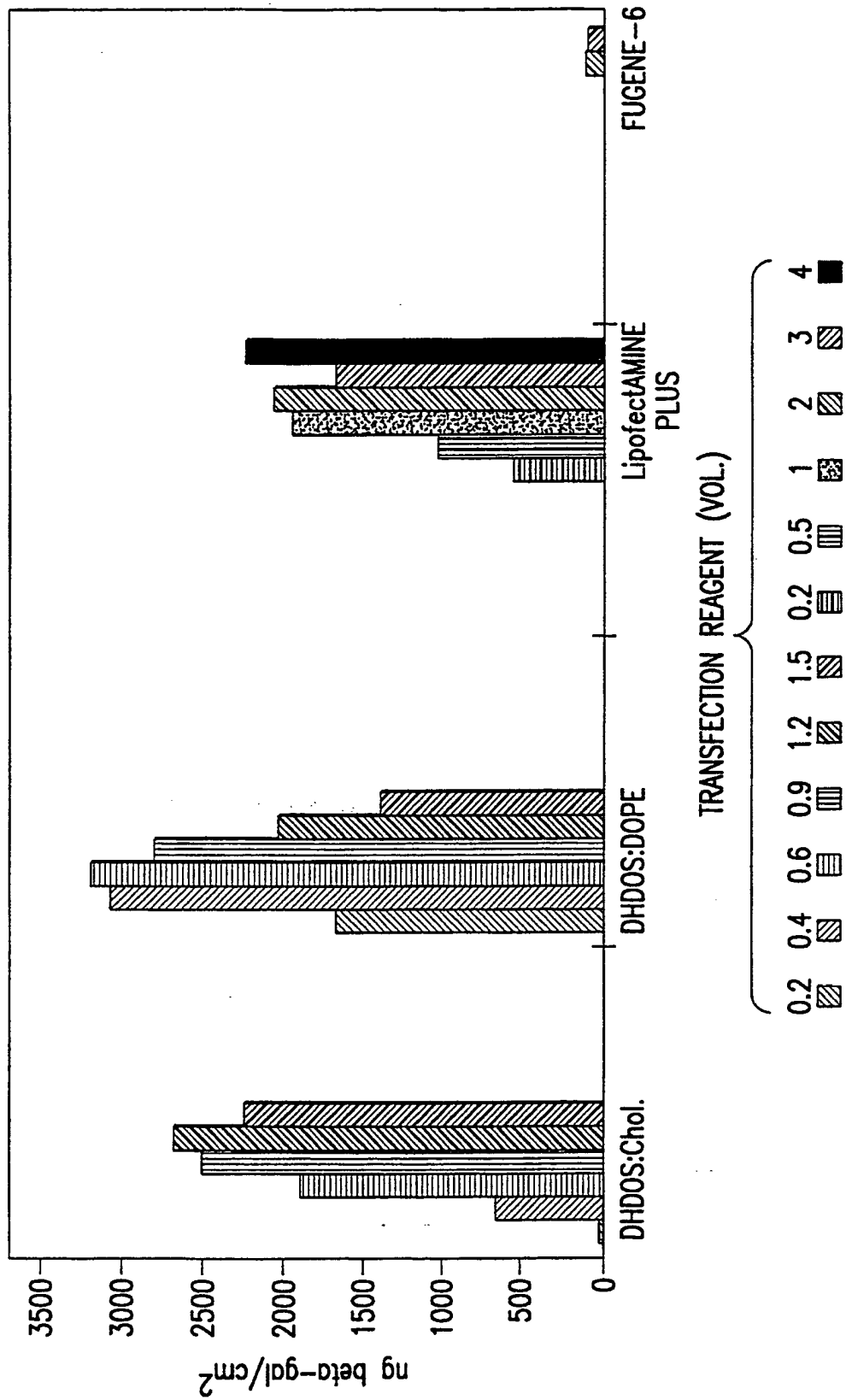


FIG.2

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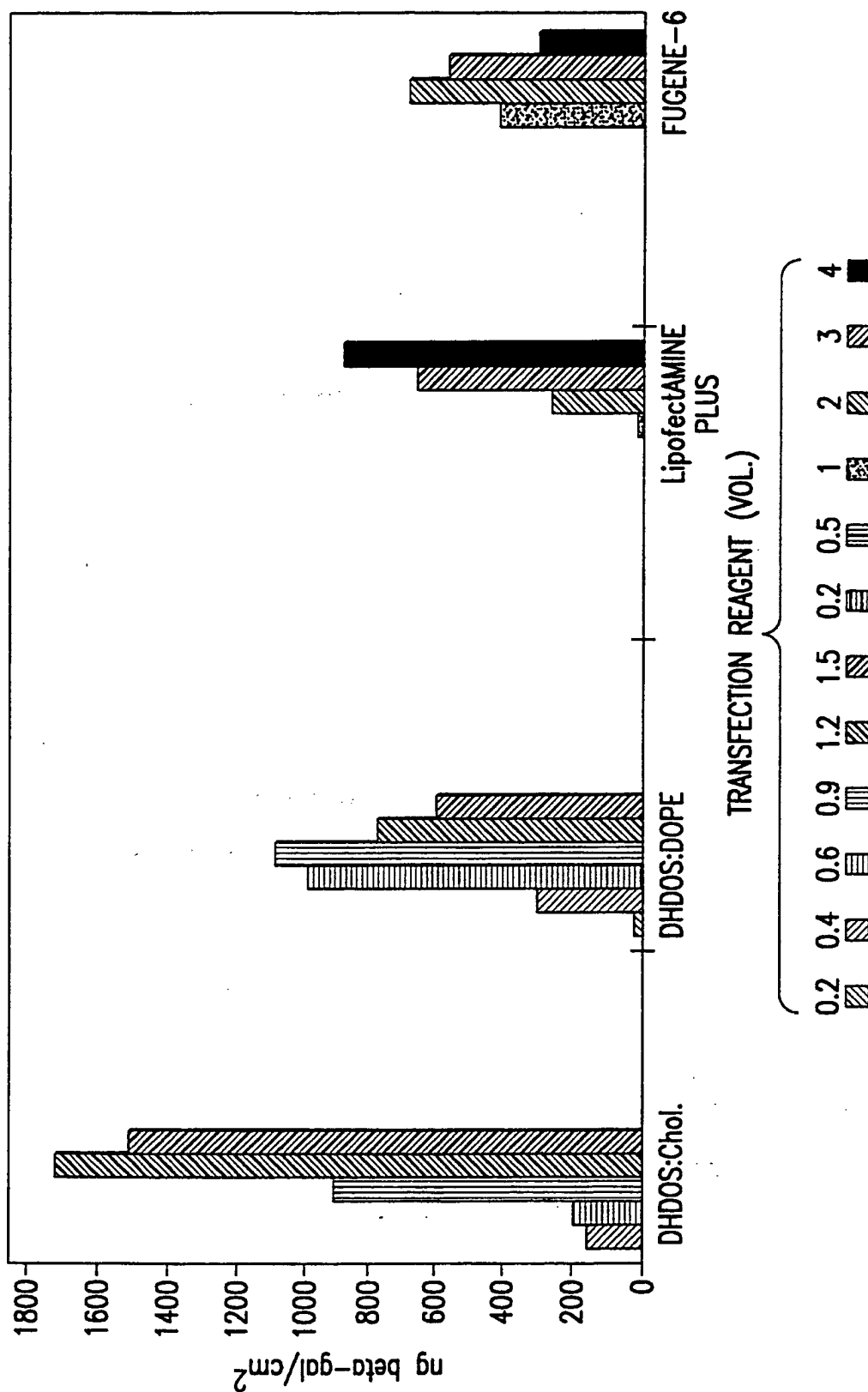


FIG.3

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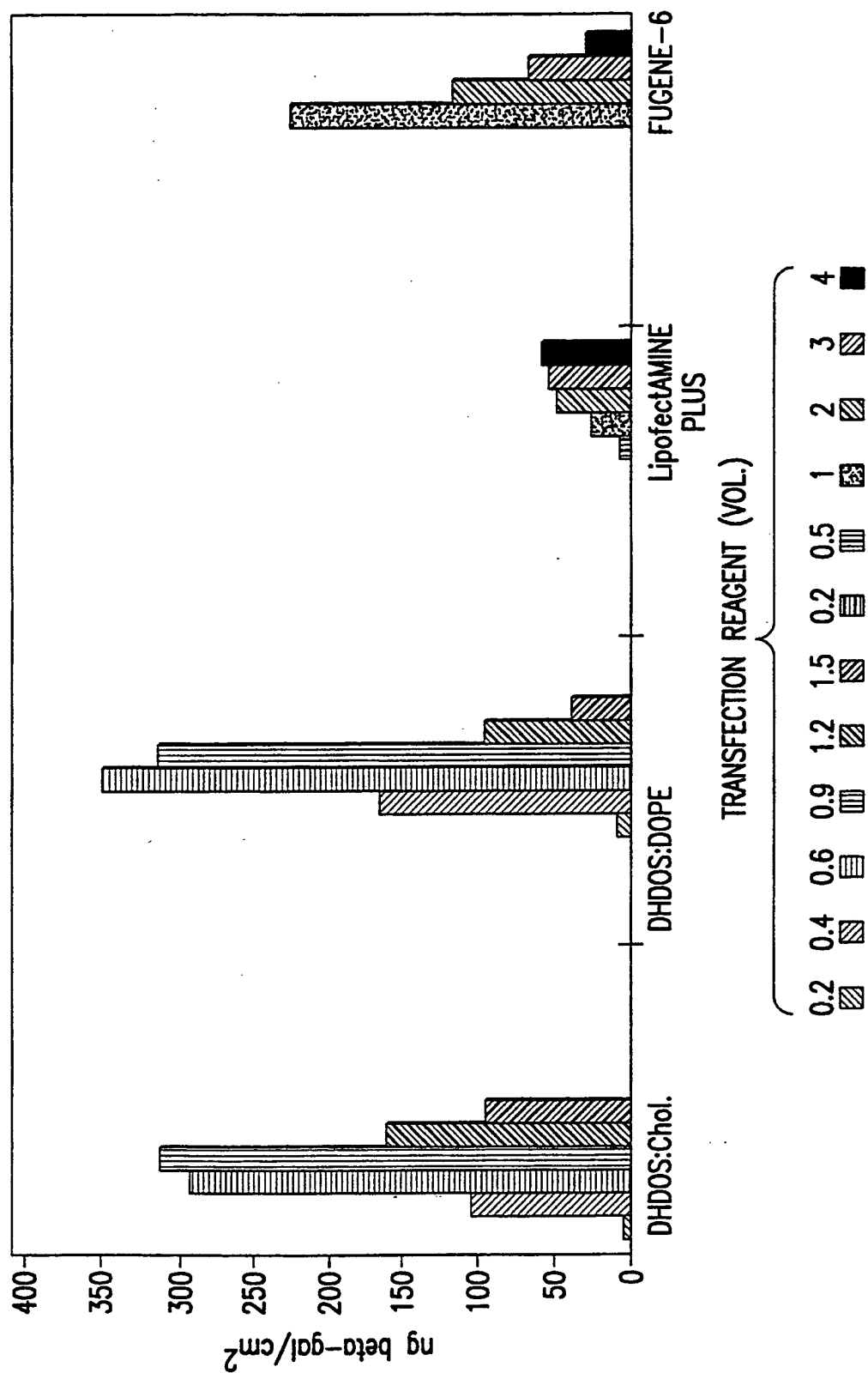


FIG.4

INTERNATIONAL SEARCH REPORT

Intern. Application No.

PCT/US 99/26825

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C07C211/64 C07C211/63 C07C229/26 C07C211/21 A61K31/14

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07C A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 42819 A (HACES ALBERTO) 20 November 1997 (1997-11-20) page 6-8 claims 1-18	1-109
X	US 5 744 335 A (WOLFF, JON A. ET AL) 28 April 1998 (1998-04-28) the whole document	1-109
	-/-	

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

16 February 2000

Date of mailing of the international search report

22/02/2000

Name and mailing address of the ISA

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Authorized officer

Bader, K

INTERNATIONAL SEARCH REPORT

Int. Application No
PCT/US 99/26825

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CHEMICAL ABSTRACTS, vol. 130, no. 8, 22 February 1999 (1999-02-22) Columbus, Ohio, US; abstract no. 91045, MCCLUSKIE, MICHAEL J. ET AL: "Direct gene transfer to the respiratory tract of mice with pure plasmid and lipid-formulated DNA" XP002129800 abstract & ANTISENSE NUCLEIC ACID DRUG DEV. (1998), 8(5), 401-414 ,	1-109
X	EP 0 846 680 A (HOFFMANN LA ROCHE) 10 June 1998 (1998-06-10) page 24-25 page 6, line 10-25	1-109
X	US 5 830 430 A (SHEN DEKANG ET AL) 3 November 1998 (1998-11-03) SEE THE EXAMPLES column 29 -column 50	1-109
X	US 5 674 908 A (HACES ALBERTO ET AL) 7 October 1997 (1997-10-07) cited in the application column 3, line 25 -column 22; claim 1	1-109
X	US 5 334 761 A (GEBEYEHU GULILAT ET AL) 2 August 1994 (1994-08-02) cited in the application column 13 -column 20	1-109
X	WO 98 40502 A (LIFE TECHNOLOGIES, INC., USA) 17 September 1998 (1998-09-17) see the examples	1-109
X	WO 98 40499 A (CAN.) 17 September 1998 (1998-09-17) see the examples	1-109
A	WO 98 02190 A (GENZYME CORPORATION, USA) 22 January 1998 (1998-01-22)	1-109
P,X	WO 99 29712 A (SMITHKLINE BEECHAM PLC, UK) 17 June 1999 (1999-06-17) see the examples	1-109
X	FR 1 567 214 A (HENKEL UND CIE. G.M.B.H.) 16 May 1969 (1969-05-16) page 1 -page 3	1

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 99/26825

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 99 26825

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Present claims 1-109 relate to an extremely large number of possible compounds. In fact, the claims contain so many options, variables, possible permutations and provisos that a lack of clarity and conciseness within the meaning of Article 6 PCT arises to such an extent as to render a meaningful search of the claims impossible. Consequently, the search has been carried out for those parts of the application which do appear to be clear and concise, namely the compounds of the specific embodiments as mentioned on page 54-65

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter. .al Application No

PCT/US 99/26825

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 9742819	A	20-11-1997	NONE	
US 5744335	A	28-04-1998	NONE	
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